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Sustained TCR Signaling Is Required for Mitogen-Activated Protein Kinase Activation and Degranulation by Cytotoxic T Lymphocytes

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Requirements for T cell activation are not fully established. One model is that receptor occupancy and down-regulation are essential for activation, and another, not necessarily mutually exclusive, model is that sustained signals are important. Here we examine the importance of signal duration in T cell activation. First, we demonstrate that immobilized, but not soluble cross-linked, Abs to CD3 stimulate degranulation by CTL. The cross-linked Abs are not deficient in their ability to signal since they stimulate the same tyrosine phosphorylation pattern as immobilized Ab, but it is very transient relative to that stimulated by immobilized Ab. Furthermore, novel decreased migratory forms of Lck occur to a significant extent only after stimulation with immobilized Abs. A dramatic difference in the duration of signals is very evident when mitogen-activated protein kinase (MAPK) activity is examined. Immobilized anti-CD3 stimulates very high levels of MAPK activation that is still detectable 1 h after stimulation. In contrast, cross-linked Ab stimulates only transient and incomplete activation of MAPK. Taken together, these results suggest that TCR engagement and induction of tyrosine phosphorylation alone are not sufficient for T cell activation and that the duration of TCR-stimulated signals is critical to attain a functional response.


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3 Abbreviations used in this paper: MAP, mitogen-activated protein; APL, altered peptide ligands; Bis, N,N’-Methylene-bis-acrylamide.

Materials and Methods

Cell lines

Murine CD8+ CTL clones, clone 11 and clone AB.1 (H-2d anti-H-2b), have been described previously (13). The clones are stimulated weekly with irradiated spleen cells from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) and used 4 to 7 days later.

Abs and reagents

The hybridoma producing 145-2C11 (anti-CD3ε) was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and PY-72 (anti-phosphotyrosine) was provided by Dr. B. Sefton (The Salk Institute, La Jolla, CA). They were grown in Protein-Free Hybridoma Medium-II (Life Technologies, Burlington, ON), and the Abs were purified by ammonium sulfate precipitation followed by either protein A or protein G chromatography, if required. Anti-MAP kinase (Erk 1) and anti-p56λκ mAbs were purchased from Transduction Laboratories (Lexington, KY).
Phospho-specific MAP kinase Ab was purchased from New England Bio-labs (Beverly, MA). Goat anti-hamster, rabbit anti-hamster, and goat anti-mouse Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PMA and A23187 were purchased from Sigma Chemical Company (Mississauga, ON) and Calbiochem (San Diego, CA), respectively. The MAP kinase kinase (MEK) inhibitor PD 98059 was purchased from Calbiochem.

Protein immobilization

Ninety-six well, flat-bottom plastic microtiter plates (Becton Dickinson, Oxnard, CA) were coated with 145-2C11 at the indicated concentration overnight at 4°C. Wells were then washed twice with PBS, blocked with 2% BSA in PBS for 30 min at 37°C, and then washed twice with PBS.

Degranulation assay

Degranulation was measured by the release of serine esterase (13). Clone AB.1 or clone 11 cells were washed three times in RPMI 1640 with 2% newborn calf serum. Cells (1.5 x 10⁶) in 150 μl of 2% FCS in RPMI 1640 were added to each well of a microtiter plate. For the soluble cross-linked Ab stimulation, cells at 10⁵ cells per ml were incubated with various concentrations of 145-2C11 for 30 min on ice, washed, and resuspended in RPMI 1640 supplemented with 2% FCS. The cells were added directly to the wells immediately after addition of either 1.0 or 10.0 μg/ml goat anti-hamster Ab. Cells were pretreated with PD 98059 for 30 min at 37°C before addition of the cells to the Ab-coated wells. For assays employing PMA (125 ng/ml) and/or A23187 (50 μM), reagents were added to cells immediately after adding the cross-linking Ab. After 4 to 5 h at 37°C, 25 μl of supernatants were tested for benzoyloxycarboxyl-L-lysine thiobenzyl ester (BLT)-esterase activity (13). Results were read at 405 nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). All samples were done in triplicate or quadruplicate, and the SD is shown.

Western blotting

CTL clones were harvested and washed in D-PBS (Life Technologies). Cells (1.5 x 10⁶) in 50 μl were stimulated as for the degranulation assay but in serum-free D-PBS. Cells were incubated at 37°C for the indicated times. Cells were lysed by the addition of 40 μl 2x Laemmli reducing sample buffer and boiled for 2 min. Whole cell lysates were loaded on either 7.5% standard (37.5:1 acrylamide:Bis) or 8.5% low Bis (56.6:1 acrylamide:Bis) SDS-PAGE, electrophoresed, and the proteins transferred to Immobilon P (Millipore Corporation, Bedford, MA) overnight at 75 mA. The MAP kinase and phospho-MAP kinase were detected using anti-phosphotyrosine (PY-72), anti-p56 Ab, or anti-Phospho MAP kinase and rabbit anti-mouse Ab. Bound Ab was detected by Enhanced Chemiluminescence (NEN, Life Science Products, Boston, MA). Results were read at 405 nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). All samples were done in triplicate or quadruplicate, and the SD is shown.

Results

CTL degranulation requires an immobilized stimulus

As we have previously demonstrated (14), immobilized Ab to CD3ε (145-2C11) or the TCR (H57-597; data not shown) stimulates a degranulation response by CTL clone AB.1 (Fig. 1). Cells were plated on microtiter wells coated with 2C11 at various concentrations, and the serine esterase release was assayed to measure degranulation. In contrast, when we performed the same assay using cross-linked Abs, we observed that soluble cross-linked Ab to either CD3ε (Fig. 1) or the TCR (data not shown) was unable to stimulate a degranulation response. Cells were incubated with soluble 2C11 at various concentrations for 30 min on ice. Cells were either used directly or cross-linked with secondary goat anti-hamster Ab at either 1.0 or 10.0 μg/ml. Degranulation significantly above that of background was not observed at a range of both primary and secondary Ab concentrations (Fig. 1, and data not shown); no cross-linking conditions were found that could stimulate degranulation above the background control of BSA alone. Ice pretreatment had no effect on the degranulation in response to immobilized anti-CD3 stimulation (data not shown).

FIGURE 1. Immobilized, but not soluble or cross-linked, anti-CD3 triggers CTL degranulation. Clone AB.1 cells (1.x 10⁵) were stimulated with immobilized anti-CD3 (IMM 2C11) at 0.05 to 20.0 μg/ml (squares). Alternatively, cells were stimulated with 0.05 to 20.0 μg/ml soluble 2C11 alone (triangles) or followed by cross-linking (XL) with goat anti-hamster Ab at 1.0 μg/ml (diamonds) or 10.0 μg/ml (circles). The blocking protein BSA is used as a background control. Cells were incubated at 37°C for 4 h, and supernatants were assayed for serine esterase release.

FIGURE 2. Kinetics of protein tyrosine phosphorylation of total cell lysates following anti-CD3 stimulation. Clone AB.1 cells (1.x 10⁵) were stimulated with soluble cross-linked (XL) 2C11 and goat anti-hamster Ab at each 10 μg/ml or with immobilized 2C11 at 20 μg/ml and incubated at 37°C for the indicated times. Cell lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting as described in Materials and Methods.

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cross-linked stimulation, as compared with 50 min for immobilized conditions. The same results are seen with anti-TCR (H57–597) stimulation by soluble cross-linked and immobilized conditions, respectively (data not shown).

The duration of tyrosine phosphorylation of several immunoprecipitated intracellular proteins follows that of the whole cell lysates (data not shown). The CTLs are therefore capable of quantitatively and qualitatively undergoing the same tyrosine phosphorylation events under both stimulation conditions, with the duration of the signal being the only obvious difference. This indicates that the immobilized stimulus allows for sustained tyrosine phosphorylation that is not achieved when soluble cross-linked stimulation is used. These results demonstrate that induction of tyrosine phosphorylation by a TCR agonist does not per se lead to a functional response.

**p56^{lck}** undergoes a mobility shift only with immobilized Ab stimulation

TCR/CD3 complex stimulation leads to the recruitment and activation of p56^{lck}. The enzymatic activity of p56^{lck} plays a pivotal role in the downstream tyrosine phosphorylation events that result in T cell activation (15, 16). It has also been shown that p56^{lck} undergoes a mobility shift to p60^{gdr} following anti-CD3 stimulation (17–19). This shift may be due to increased serine phosphorylation of p56^{lck} (17, 18), the function of which is not clearly understood. To determine whether these stimulation conditions lead to this migratory shift, CTL clones were stimulated with either soluble cross-linked or immobilized anti-CD3. Total cell lysates were separated by SDS-PAGE using 8.5% low Bis gels, which have higher resolution in the 40- to 60-kDa range, and probed with a mAb to p56^{lck}. The resulting immunoblot (Fig. 3) indicates that, following soluble cross-linked anti-CD3 stimulation, there is a dominant form of p56^{lck} (arrow 1) and a minor higher m.w. form (arrow 2). p56^{lck} from CTLs stimulated with immobilized anti-CD3 undergo a more extensive mobility shift to the higher m.w. form (arrow 2). Also, two higher m.w. p56^{lck} forms (arrows 3 and 4) are present at 30 min following immobilized Ab stimulation, and they are sustained for at least 60 min.

**Failure to induce degranulation by soluble cross-linked Ab is not restored with increased intracellular Ca$^{2+}$ or protein kinase C (PKC) activation**

Recently it was reported that the Ca$^{2+}$ flux in T cells is more sustained following stimulation with surface-attached ligands than soluble ligands (20). One possibility is that the soluble cross-linking conditions do not stimulate a sustained Ca$^{2+}$ flux, and we have shown that Ca$^{2+}$ is required for degranulation by this CTL clone (21). If this is the case, then we should be able to restore degranulation in the CTL clones with a Ca$^{2+}$ ionophore. Adding the Ca$^{2+}$ ionophore A23187 and the phorbol ester PMA together triggers CTL degranulation whereas either alone does not (Fig. 4). Treatment of cells with A23187 along with soluble cross-linked Ab does not induce degranulation in the CTL clones. Addition of PMA following soluble cross-linking Ab stimulation also does not induce degranulation as compared with the control treatment with both A23187 and PMA. This would indicate that the defect in CTL degranulation is not simply a lack of increased intracellular Ca$^{2+}$ or protein kinase C (PKC) activation but suggests that both pathways are insufficiently triggered by cross-linked Abs.

**Sustained MAP kinase activation requires an immobilized stimulus**

Since both stimulation conditions induce tyrosine phosphorylation in the CTL clones, whereas degranulation is not triggered with soluble cross-linked Ab, we examined another downstream effector outcome following TCR complex stimulation. We examined the consensus MAP kinase activation pathway. Activation of MAP kinase is required for CTL degranulation since PD 98059, an inhibitor of this pathway, decreases the serine esterase release response (Fig. 5). Previous data in fibroblasts indicated that MAP kinase activation was dependent on cell anchorage and that greater MAP kinase activation occurred in cells adhering to fibronectin than those in suspension (22). It is possible that similar criteria are
FIGURE 6. Kinetics of MAP kinase activation following immobilized and soluble cross-linked anti-CD3 stimulation. Clone AB.1 cells (1.5 × 10^5) were stimulated with soluble cross-linked (XL) 2C11 at 10 μg/ml and rabbit anti-hamster Ab at 5 μg/ml or immobilized 2C11 at 20 μg/ml and incubated at 37°C for the indicated times. The control lanes (C) are from cells incubated on BSA for 20 min. Whole cell lysates were run on a 15% low-Bis gel (upper panel) or a 10% SDS-PAGE gel (lower panel). The samples were then immunoblotted with anti-MAP kinase (upper panel) or anti-phospho-specific MAP kinase (lower panel) as described in Materials and Methods. pp42 indicates the position of the shifted p42.

necessary for T cells even though T cells are not considered adherent. CTL clones were stimulated as described above, and whole cell lysates were run on a low-Bis 15% SDS-PAGE and immunoblotted for MAP kinase (Fig. 6). MAP kinase undergoes decreased mobility due to phosphorylation, which can be used to assess the extent of activation of the enzyme when low-Bis gels are employed. Following soluble cross-linked Ab stimulation, there is transient and partial activation of MAP kinase for about a 5-min duration as indicated by the mobility shift. Following immobilized Ab stimulation, the mobility shift is evident at 20 min, and some shifted material is present even at 60 min. Furthermore, the extent of MAP kinase activation is significantly greater after stimulation with immobilized Abs. These results were confirmed using a phospho-specific MAP kinase Ab that recognizes only catalytically activated p42 and p44 MAP kinases (Fig. 6, bottom panel). Again, there is only very transient and limited MAP kinase activation after induction with cross-linked Ab whereas extensive and prolonged MAP kinase activation is induced with immobilized Ab. Thus, for sustained MAP kinase activation, an immobilized stimulus is required.

An intact cytoskeleton is required for sustained tyrosine phosphorylation

Upon incubation with soluble cross-linked Ab, there will be a uniform distribution of Ab on the cell surface with most receptors engaged as compared with the more polarized engagement with immobilized Ab. It is plausible that the tyrosine phosphorylation induced with immobilized anti-CD3 requires an intact cytoskeleton, possibly for surface receptor redistribution, to be sustained. We hypothesized that inhibiting cytoskeleton assembly with cytochalasin E treatment would alter the tyrosine phosphorylation differently in the cells stimulated in the two different ways. CTL clones were treated with cytochalasin E before stimulation at 37°C for various times, and tyrosine phosphorylation of total cellular proteins was examined. Figure 7 shows that cells stimulated with immobilized 2C11 had a near complete inhibition of protein tyrosine phosphorylation in the presence of cytochalasin E (right) as compared with the normal pattern of tyrosine phosphorylation (left). In contrast, cells stimulated with soluble cross-linked 2C11 undergo the same tyrosine phosphorylation events in the absence (left) and presence (right) of cytochalasin E (Fig. 7). Consequently, in contrast to the immobilized stimulus, the soluble cross-linked Ab-induced phosphorylation is not inhibited by cytoskeleton disruption.

FIGURE 7. An intact cytoskeleton is required for tyrosine phosphorylation induced by immobilized but not by cross-linked anti-CD3. Clone AB.1 cells (1.5 × 10^5) were stimulated with immobilized 2C11 at 20 μg/ml or with soluble cross-linked (XL) 2C11 at 10 μg/ml and goat anti-hamster Ab at 10 μg/ml for the indicated times in the absence (left) or presence (right) of 10 μM cytochalasin E. Cell lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting as described in Materials and Methods.

Discussion

We have shown that the manner in which you stimulate CTLs, by an immobilized or a soluble cross-linked Ab to the TCR/CD3 complex, results in dramatically different outcomes. Both stimulation conditions trigger qualitatively and quantitatively similar patterns of cellular protein tyrosine phosphorylation, but of different duration. The phosphorylation induced by soluble cross-linked conditions is transient whereas it is sustained when induced by immobilized stimulation. Although soluble cross-linked Abs can trigger tyrosine phosphorylation, degranulation is not stimulated. One may speculate that the sustained phosphorylation is a necessary prerequisite for further downstream effector functions such as degranulation. The immobilized Ab is also necessary to achieve sustained MAP kinase activation and to induce a mobility shift in p56^{Lck}. It is therefore evident that tyrosine phosphorylation is not necessarily an indicator of a functional response. Clearly not all stimulation conditions are equal; when cells are stimulated with soluble Ab, the signaling results must be interpreted carefully since a functional response may not be stimulated.

Valitutti et al. (10, 11) have shown that the effector responses elicited from CTLs can be uncoupled from one another, and this is attributed to different levels of TCR occupancy. Glickstein et al. demonstrated that anti-CD3-stimulated apoptosis and cytokine secretion by a Th hybridoma can be uncoupled from each other by using immobilized vs soluble Ab (23). It has also been suggested that T cells have the ability to count the number of TCRs triggered with MHC/peptide (24), and, in the absence of threshold TCR engagement, there is no cell activation. Although our data support that a minimum TCR engagement at the cell surface is necessary for T cell activation, our system suggests that there is more to
activating T cells than simple receptor occupancy, since stimulation with soluble cross-linked Ab will result in essentially all the TCR complexes being engaged. Consistent with our results, it has been shown that prolonged TCR/CD3 engagement is needed for a sustained Ca\(^{2+}\) flux, which is required for T cell activation (25).

We propose that, under our immobilized Ab conditions, there is a sustained Ca\(^{2+}\) flux, which is required for T cell activation (25). We suggest that, under our immobilized Ab conditions, there is a sustained Ca\(^{2+}\) flux, which is required for T cell activation (25). We speculate that, under immobilized stimulus conditions, there is a sustained Ca\(^{2+}\) flux, which is required for T cell activation (25). We conclude that the cytoskeleton is required for the subsequent sustained phosphorylation induced by immobilized Ab and that this is not a requirement for the transient phosphorylation induced by soluble cross-linked Ab (Fig. 7). We predict that the cytoskeleton is required for cell spreading on the Ab-coated surface and engagement of sufficient receptors for spreading stimulus (25). As already discussed, however, TCR occupancy alone is not sufficient for T cell activation, and we conclude that the cytoskeleton is required for the subsequent sustained phosphorylation. The cytoskeleton, either directly or indirectly, is likely needed for the assembly of signaling molecules into multimeric complexes necessary for activation and subsequent effector functions such as degranulation.

Our data show that MAP kinase is activated only transiently with cross-linked Ab but, with immobilized Ab MAP kinase, is activated to a greater extent, and the activation is sustained. Based on a number of studies in neuronal cells, Marshall has proposed that essentially all of the receptors were “occupied,” yet no degranulation response was observed. On the other hand, if the Abs were immobilized, which does not necessarily change the extent of TCR occupancy, a degranulation response was observed. The striking difference between these two stimulation conditions is that the duration of a number of signals that we measured was significantly longer when immobilized Abs were used. Taken together, these results suggest that the duration of the response, which likely involves cytoskeletal rearrangements, dictates whether a response ensues. This observation can have significant implications for vaccine design since triggering the TCR is not sufficient to guarantee a response, but the duration of the response must also be sustained.

Acknowledgments

We thank Dr. Kevin Kane for helpful discussions and for critically reviewing the manuscript and acknowledge the significant input of Dr. Ellen Shibuya in helping us establish the MAP kinase shift assay in our laboratory.

References


Previous evidence has shown that the cytoskeleton is necessary for T cell effector functions (32) and that this may be required to sustain the intracellular signals (10). We found that an intact cytoskeleton is required for the sustained tyrosine phosphorylation induced by immobilized Ab and that this is not a requirement for the transient phosphorylation induced by soluble cross-linked Ab (Fig. 7). We predict that the cytoskeleton is required for cell spreading on the Ab-coated surface and engagement of sufficient receptors for spreading stimulus. As already discussed, however, TCR occupancy alone is not sufficient for T cell activation, and we conclude that the cytoskeleton is required for the subsequent sustained phosphorylation. The cytoskeleton, either directly or indirectly, is likely needed for the assembly of signaling molecules into multimeric complexes necessary for activation and subsequent effector functions such as degranulation.

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