Live Cell Fluorescence Imaging of T Cell MEKK2: Redistribution and Activation in Response to Antigen Stimulation of the T Cell Receptor

Brian C. Schaefer,1,7 Margaret F. Ware,2 Philippa Marrack,1,3,4,5 Gary R. Fanger,2 John W. Kappler,1,6 Gary L. Johnson,1,6 and Colin R. F. Monks2
1Howard Hughes Medical Institute and Department of Medicine
2Program in Molecular Signal Transduction Division of Basic Sciences National Jewish Medical and Research Center Denver, Colorado 80206
3Departments of Biochemistry and Molecular Genetics
4Department of Immunology
5Department of Medicine
6Department of Pharmacology University of Colorado Health Sciences Center Denver, Colorado 80220
7To whom correspondence should be addressed (e-mail: schaeferb@njc.org).

Summary

T cell activation requires engagement of the T cell receptor (TCR) at the interface of conjugates formed with antigen-presenting cells. TCR engagement is accompanied by a redistribution of specific signaling molecules to the cytoplasmic region of the TCR complex. In this study, immunocytochemistry and live cell fluorescence imaging demonstrate that T cell MEK kinase 2 (MEKK2) is translocated to the T cell/antigen-presenting cell interface in response to antigen activation. MEKK2 translocation occurs more rapidly as the antigen concentration is increased. Biochemical activation of MEKK2 follows TCR stimulation, and expression of a dominant-negative MEKK2 inhibits TCR-mediated conjugate stabilization and ERK and p38 MAP kinase phosphorylation. Live cell fluorescence imaging thus enables characterization of signal transducers that are dynamically translocated following TCR engagement.

Introduction

MEK kinases (MEKKs) are a family of serine-threonine kinases that have been shown to regulate mitogen-activated protein kinase (MAPK) cascades, including the ERK, p38, and JNK pathways (Fanger et al., 1997; Widmann et al., 1999). In MAPK cascades, the MEKKs function as MAPK kinase kinases (MAPKKKs) that activate a MAPK kinase (MAPKK), and the MAPKK then activates a MAPK. Many MAPKKKs, including the MEKKs, are capable of activating more than one MAPK pathway. Additionally, most cell types express multiple MAPKKKs, which, in transfection/overexpression experiments, often appear to be functionally interchangeable (Widmann et al., 1999). However, emerging evidence suggests that MAPK cascades are physically compartmentalized by scaffolding proteins that group specific MAPK signal transducers (Schaeffer et al., 1998; Whitmarsh et al., 1998). Such subcellular organization may thus provide a means by which a receptor may be coupled to a specific MAPKKK, MAPKK, and MAPK.

The transduction of signals from the T cell receptor (TCR) involves multiple cascades of many different signaling proteins that activate diverse targets, including MAPKs. Increasing evidence is accumulating that these signaling events are accompanied by a redistribution of some or all of the participating molecules into discrete, macromolecular signaling units. In T cells, antigen stimulation results in clustering of multiple cell surface molecules into discrete domains (Bonaldez et al., 1998; Monks et al., 1998; Wulfing and Davis, 1998; Wulfing et al., 1998; Penninger and Crabtree, 1999) and the concomitant translocation of signaling molecules to the cytoplasmic face of the clustered receptors (Monks et al., 1997).

Such protein redistribution represents a consequence of active signaling that can be visualized in fixed cells using immunocytochemistry (Monks et al., 1997, 1998). Redistribution in response to a specific signal provides evidence that the redistributed protein plays an active role in transducing that signal. It is therefore possible to use immunocytochemistry as a screening method to determine if a given protein is likely to be involved in the response to a signal by assessing its redistribution. A recently developed tool for studies of this type is green fluorescent protein (GFP), which can be used as a covalently bound tag to allow the characterization of dynamic protein redistribution in single cells in real time. Thus, analysis of the subcellular redistribution of MAPK signal transducers in response to the ligation of the TCR should provide an accurate means of determining whether a particular MAPK transducer is involved in the delivery of antigen-mediated signals.

We have used digital fluorescence imaging of fixed and live T cell clones to determine whether MEKKs are involved in transducing antigen-dependent signals in T cells. We have found that endogenous T cell MEKK2, but not MEKK1 or MEKK3, translocates to the cytoplasmic face of the interface with antigen-loaded presenting cells. Experiments employing an MEKK2-GFP fusion protein show that translocation of MEKK2 occurs within seconds of exposure to presenting cells loaded with a highly stimulatory dose of antigen. However, as antigen becomes limiting, translocation of MEKK2-GFP does not occur until several minutes after initial contact with the presenting cell. MEKK2 is activated by TCR stimulation, and biochemical data indicate that MEKK2 activates T cell adhesion, as well as the ERK and p38 MAPK cascades. However, MEKK2 does not appear to directly regulate IL-4 production in response to antigen stimulation. Although a complete pathway for TCR-mediated MEKK2 activation remains to be defined, experimental evidence suggests that PI3-kinase may be an upstream modulator of MEKK2 activation. MEKK2 is thus a newly identified transducer of TCR signals that participates in the activation of specific antigen-regulated pathways.
Results

Translocation of T Cell MEKK2 in Response to Antigen Stimulation

Because MAPK cascades are known to be involved in multiple aspects of T cell activation (Cantrell, 1996), experiments were performed to determine if MEKK family members are involved in early T cell signaling. Conjugates between T cells and antigen-pulsed presenting cells were assayed at early timepoints for MEKK protein redistribution via digital immunofluorescence microscopy (Munks et al., 1997, 1998). The D10 T cell clone, which reacts with I-A^k plus a peptide from conalbumin (Kaye et al., 1983), was incubated with I-A^k-bearing CH12.LX B cells (APC) that had been either untreated or pulsed overnight with conalbumin. The T cell/APC conjugates that formed were fixed and stained for either MEKK1 or MEKK2 and digitally imaged. Figure 1A shows that MEKK2 in the T cell translocated to the contact site between the T cell and APC, providing the APC had been pulsed with antigen, whereas MEKK1 remained distributed equally throughout the T cell cytoplasm.

To verify these data, D10 T cells were infected with a retrovirus coding for MEKK2 fused in frame to green fluorescent protein (MEKK2-GFP), and a polyclonal cell line was established. Detection of GFP allowed assessment of the distribution of the MEKK2-GFP fusion protein in live cells. The D10/MEKK2-GFP line wasaging data, T cell/APC conjugates were synchronously assessed for either MEKK1 or MEKK2 and digitally imaged. Figure 1A shows that MEKK2 in the T cell translocated to the contact site between the T cell and APC, providing the APC had been pulsed with antigen, whereas MEKK1 remained distributed equally throughout the T cell cytoplasm.

To establish how antigen concentration affects the kinetics of MEKK2 translocation, live cell imaging experiments were performed on conjugates of D10/MEKK2-GFP T cells and APC, which had been pulsed overnight with various concentrations of antigen. As shown in Figure 1A, translocation of MEKK2-GFP occurred within 10 sec after exposure of T cells to APC that were loaded with 500 µg/ml conalbumin. At 20 µg/ml conalbumin, translocation was delayed, requiring approximately 40 sec following initial APC contact. When antigen concentration was lowered to 4 µg/ml, there was a delay of approximately 3 to 5 min before MEKK2-GFP translocation was observed. To further support the live cell imaging data, T cell/APC conjugates were synchronously initiated by brief centrifugation and were fixed 10 or 60 min later. Based on the data in Figure 2A, we expected that almost all conjugates at high antigen concentration (500 µg/ml) would also have translocated MEKK2-GFP but that this percentage would diminish as the antigen concentration was lowered, and that no translocations would be observed in the absence of antigen. This is in fact what was observed (Figure 2B). Significantly, the most substantial drop in translocation percentage occurred between 4.0 and 0.8 µg/ml conalbumin, which is the range of concentration in which antigen becomes limiting for functional responses (see Figure 4A).

At 60 min, the percentage of conjugates with MEKK2 translocations was generally less than at 10 min, suggesting that the enrichment of MEKK2 at the T cell/APC interface may be reversed before conjugate dissociation. This observation may suggest that MEKK2-GFP translocation is of shorter duration at lower antigen concentration (Figures 2A and 2B). Although some of our live cell images seem to support the idea that MEKK2 translocation is transient, cell movement during imaging experiments of this duration complicates their interpretation. The data in Figures 2A and 2B do, however, strongly suggest that the rate and duration of MEKK2-GFP translocation is directly related to the number of specific antigen/MHC complexes present on the APC.

TCR-Dependent Activation of MEKK2

To determine whether T cell MEKK2 is activated by TCR engagement, the kinase activity of the MEKK2-GFP fusion protein in the D10/MEKK2-GFP line was tested using a GST-SEK1 substrate. Figure 3A shows that the kinase activity of MEKK2-GFP from D10 cells that had been incubated with fixed, conalbumin-pulsed APC was 4-fold higher than that of MEKK2-GFP isolated from D10 cells incubated without APC or with fixed APC that had
Live Cell Fluorescence Imaging of MEKK2

Figure 1. Translocation of T Cell MEKK2 in Response to Antigen-Loaded APC

(A) D10 T cells were mixed with APC loaded with no antigen (no ag) or with 500 \( \mu \)g/ml conalbumin for 10 min at 37°C. Conjugates were bound to coverslips, and MEKK2 and MEKK1 were detected by immunofluorescence microscopy using either rabbit anti-MEKK2 or rabbit anti-MEKK1 antibodies and a Cy3-conjugated anti-rabbit secondary reagent. The upper panels are Nomarski views of the same conjugates shown in the lower panels, which were imaged by immunofluorescence and digitally deconvolved. In each panel, the APC is the upper cell and the D10 T cell is the lower cell.

(B) An MEKK2-GFP retrovirus was used to introduce an MEKK2-GFP fusion protein into D10 T cells. D10/MEKK2-GFP T cells were mixed with equal numbers of conalbumin (500 \( \mu \)g/ml)-loaded APC, and real-time fluorescent images were acquired. Exposures of 10 sec each were acquired at 30 sec intervals over a period of 36 min. Selected panels at the indicated timepoints are shown (APC and T cell are same orientation as in [A]). Three similar series can be viewed as Quicktime movies at http://www.intelligent-imaging.com/njcfigs.html. Some of these additional images show that multiple interactions result in multiple translocations, demonstrating that the observed translocations do not simply represent reorientation of the golgi and MTOC.

(C) D10 T cells expressing MEKK2-GFP, dnMEKK2-GFP, or MEKK3-YFP were mixed for 10 min at 37°C with APC that had been loaded overnight with no antigen (no ag) or with 500 \( \mu \)g/ml conalbumin. Conjugates were bound to coverslips, fixed, and imaged as described in the Experimental Procedures. Upper panels are Nomarski images, and bottom panels are digitally deconvolved fluorescence images.

(D) Conjugates prepared as described in (C) were imaged in 0.2 \( \mu \)m steps through the entire cell volume (10–15 \( \mu \)m). The data shown represent the integral of the fluorescence intensity of the manually defined contact site over the total value of the cell. Error bars represent the standard deviation. The manually defined contact sites represent 6.9 ± 2.8% of the total cell volume. The relative enrichment of MEKK2-GFP at the manually defined contact site is 1.03 ± 0.14 when stimulated with APC that had not been pulsed with antigen (no ag) and 2.38 ± 0.58 when stimulated with APC that had been loaded with 500 \( \mu \)g/ml conalbumin. The relative enrichment of MEKK3-YFP at the manually defined contact site is 1.08 ± 0.11. The digitally defined contact site (calculated only for MEKK2-GFP translocation in response to antigen stimulation; see Experimental Procedures) represents approximately 1.25% of the total cell volume and 9% of the total cellular MEKK2-GFP fluorescence. The relative enrichment of MEKK2-GFP at the digitally defined contact is 6.75 ± 1.76.

not been pulsed with antigen. As expected, no phosphorylation of GST-SEK1 was observed when cells that express the kinase-inactive mutant, dnMEKK2-GFP, were used (data not shown). Thus, engagement of D10 T cells by antigen-pulsed APC activates MEKK2-GFP. Furthermore, the observed activation was TCR mediated, because it was both antigen specific and accomplished via the use of fixed APCs, which are incapable of
Figure 2. Effect of Antigen Concentration on Kinetics of MEKK2-GFP Translocation

(A) Live cell imaging is described in the Experimental Procedures. APC were loaded with the indicated concentrations of conalbumin. In each panel, the APC is the upper (dark) cell and the D10 T cell is the lower (bright) cell.

(B) T cell/APC conjugates were prepared, bound to coverslips, and fixed as described in the Experimental Procedures. Conjugates were identified visually using Nomarski optics, and fluorescence imaging was then used to determine whether there was enrichment of MEKK2-GFP at the T cell/APC contact site. A total of 100–160 conjugates were examined over two separate experiments for each conalbumin loading concentration, and the percentage of conjugates that exhibited MEKK2-GFP translocation is reported.


A Dominant-Negative Form of MEKK2 Blocks TCR Activation of the ERK and p38 MAPKs but Not the JNK MAPK Pathway

Previous studies of MAP kinase activation in T lymphocytes have demonstrated that cross-linking of the TCR results in rapid activation of the ERK and p38 MAPK pathways, whereas activation of the JNK MAPK pathway requires cross-linking of both the TCR and CD28 (Whitehurst et al., 1992; Su et al., 1994; Cantrell, 1996; Li et al., 1996; Salmon et al., 1997). Moreover, MEKK2 has been shown to be a MAPK kinase kinase that can activate the MAPK kinases MEK-1 and MEK-2, which are the kinases directly upstream of the ERK MAPKs (Fanger et al., 1997). Thus, to establish whether MEKK2 has a role in T cell MAPK activation, we used the polyclonal D10/dnMEKK2-GFP line to look for evidence of inhibition of signals known to be downstream of TCR ligation.

D10/MEKK2-GFP, D10/dnMEKK2-GFP, and noninfected D10 cells were activated by treatment with the stimulatory anti-TCR monoclonal antibody, 3D3 (Kaye et al., 1983). The cells were then lysed and assayed by immunoblot using antibodies specific for activated, phosphorylated ERK-1 and ERK-2 and p38 MAPK. ERK-1 and ERK-2 were phosphorylated in D10 and D10/MEKK2-GFP T cells in response to treatment with the anti-TCR antibody (Figure 3B). ERK phosphorylation was substantially blocked by the MEK inhibitor, PD98059 (data not shown). In contrast, the stimulatory anti-TCR antibody did not induce phosphorylation of ERK-1 and ERK-2 in cells expressing dnMEKK2-GFP (Figure 3B).
products were separated by SDS-PAGE, and 32P-labeled GST-cJun was quantified using a phosphorimager. Relative phosphorylation units are indicated beneath each lane.

**Figure 3. Biochemical Analysis of Activities of MEKK2-GFP and dnMEKK2-GFP in D10 T Cells**

(A) D10/MEKK2-GFP T cells were incubated at 37°C for 10 min alone (control) or with paraformaldehyde-fixed APC that had previously been untreated (APC + no ag) or loaded overnight with 500 μg/ml conalbumin (APC + con alb). Immunoprecipitated MEKK2-GFP was incubated with purified recombinant GST-SEK1 and γ-[32P]-ATP, and products were separated by SDS-PAGE. To measure MEKK2 kinase activity, phosphorimage analysis was performed to quantitate phosphorylated GST-SEK1. Relative phosphorylation units are indicated beneath each lane.

(B) D10 T cells, D10/MEKK2-GFP T cells, or D10/dnMEKK2-GFP T cells were mock treated or activated for 5 min at 37°C with the D10-specific, TCR-activating monoclonal antibody 3D3. Protein extracts were prepared and separated on SDS-PAGE gels as described in (A). Phosphorylated p38 MAPK (phos p38) was detected with an anti-phospho-p38 MAPK antibody (upper panel). This blot was then stripped and blotted with an anti-p38 antiserum, as shown in the lower panel.

(C) D10 T cells, D10/MEKK2-GFP T cells, or D10/dnMEKK2-GFP T cells were mock treated or incubated for 5 or 10 min at 37°C with ionomycin (iono). Cells were disrupted and cJun NH2-terminal kinase (JNK) activity was assayed using a GST-c-Jun kinase assay. Reaction products were separated by SDS-PAGE, and 32P-labeled GST-cJun was quantified using a phosphorimager. Relative phosphorylation units are indicated beneath each lane.

(D) D10 T cells, D10/MEKK2-GFP T cells, or D10/dnMEKK2-GFP T cells were mock treated (control) or activated for 5 min at 37°C by the addition of PMA and ionomycin (PMA + iono). Cells were disrupted and cJun NH2-terminal kinase (JNK) activity was assayed using a GST-cJun kinase assay. Reaction products were separated by SDS-PAGE, and 32P-labeled GST-cJun was quantified using a phosphorimager. Relative phosphorylation units are indicated beneath each lane.

Stimulation of D10 or D10/MEKK2-GFP cells with the anti-TCR 3D3 antibody also rapidly induced p38 phosphorylation (Figure 3C). Expression of dnMEKK2-GFP inhibited this phenomenon. This result was somewhat surprising because transfection analyses in other cell types have not demonstrated p38 activation in response to transient overexpression of full-length MEKK2 or its kinase domain (Fanger et al., 1997; Widmann et al., 1999).

In contrast to the ERK and p38 MAPK pathways, PMA/ionomycin-mediated activation of JNK was not significantly inhibited by expression of dnMEKK2-GFP (Figure 3D). Therefore, dnMEKK2-GFP selectively inhibits the ERK and p38 MAP kinase pathways that are activated by engagement of the TCR alone but does not inhibit the JNK pathway, which also requires costimulatory signals. Activation of MEKK2 is thus downstream of TCR ligation and is not dependent upon the cross-linking of additional cell surface molecules.

**MEKK2 Influences T Cell Adhesion**

To assay the effects of dnMEKK2-GFP on T cell function, D10 and its MEKK2-GFP infectants were tested for their ability to secrete IL-4 in response to antigen and APC. Figure 4A demonstrates that expression of dnMEKK2-GFP reduced the sensitivity of D10 cells to low concentrations of antigen. In time-lapse microscopy studies, we observed that expression of dnMEKK2-GFP by D10 both slowed the formation and reduced the number of conjugates that D10 formed with antigen-pulsed APC (data not shown). To address the possibility that D10/dnMEKK2-GFP T cells might have reduced levels of one or more cell surface proteins required for efficient interaction with APCs, FACS analyses were used to measure surface expression of molecules that contribute to conjugate formation. All but one of the proteins monitored (TCRβ, CD4, CD28, LFA-1, ICAM-1, and CD2) varied less than 2-fold in their levels on the three D10 cell lines (data not shown). The one notable exception was CD28, which, in comparison to D10 T cells, was substantially decreased on the D10/MEKK2-GFP line and modestly increased on the D10/dnMEKK2-GFP line. Because CD28 probably contributes to regulation of T cell/B cell interactions, this CD28 phenotype did not correlate with the observed decrease in specific conjugate formation of the D10/dnMEKK2-GFP T cells.

The fact that expression of dnMEKK2-GFP reduced the ability of T cells to respond to antigen and to form conjugates suggested that dnMEKK2-GFP affected a feature of T cell activation that contributes to both of these activities. Previous studies of T cell activation have shown that antibodies to T cell adhesion proteins, such as the integrin LFA-1, have effects similar to those shown here with dnMEKK2-GFP expression (Davignon et al., 1981; Golde et al., 1986). Engagement of TCRs on T cells leads to a rapid and transient increase in the avidity of T cell LFA-1 for its ligands on other cells (Dustin and Springer, 1989). In turn, this increase in avidity contributes to the ability of activated T cells to bind to target cells and produce cytokines. Anti-LFA-1 has a modest effect on the ability of T cells to secrete cytokines in response to antigen and a more dramatic effect on their ability to form conjugates with antigen-pulsed APCs.
Figure 4. Effect of Expression of MEKK2-GFP and dnMEKK2-GFP on Antigen-Dependent T Cell Responses

(A) The indicated D10 T cells were mixed with APC that had been loaded overnight with the indicated concentrations of conalbumin protein. Supernatants were harvested following 6 hr incubation at 37°C. IL-4 concentrations were measured by capture ELISA and calibrated to a recombinant IL-4 standard curve. Error bars represent SEM, and the majority are contained within the symbols.

(B) APC were labeled with the red dye PKH-26 and then loaded with the indicated concentration of conalbumin protein or with no antigen. The D10 and D10/dnMEKK2-GFP T cell lines were labeled with the green dye CFSE. Each D10 T cell line was then incubated for at least 30 min at 37°C with no antibody, or 100 µg/ml of blocking antibodies directed against LFA-1 or CD4. For each sample, T cells were mixed with an equal number of APC and incubated for exactly 16 min at 37°C. Nonspecific aggregates were disrupted by vortexing, and samples were analyzed by flow cytometry. A representative set of two-dimensional plots of D10/MEKK2-GFP T cells (green) versus PKH-26-labeled APC (red) is shown. In this example, T cells were preincubated with no antibody or anti-LFA-1 antibody, and APC were loaded with conalbumin (20 µg/ml) or no antigen. The number in each plot is percent conjugates.

(C and D) Tabulation of data collected from flow cytometry experiments described in (B). In each graph, percent (%) conjugates versus concentration of conalbumin used for APC loading is shown. In (C), each untreated cell line (no antibody) is shown on a single graph. In (D), each cell line is shown on a separate graph, with all three treatment groups (no antibody, anti-LFA-1, and anti-CD4) shown. Error bars represent SEM, and the majority are contained within the symbols.

(Davignon et al., 1981; Golde et al., 1986; Dustin and Springer, 1989). Interestingly, there is at least one report that the ERK MAPK pathway contributes to TCR regulation of integrin avidity (Mobley et al., 1996).

Several additional findings suggest a relationship between the effects of dnMEKK2-GFP and activation of LFA-1. For example, both antigen-stimulated MEKK2-GFP translocation and increases in LFA-1 adhesion to its ligand, ICAM-1, occur with rapid kinetics and have a duration of at least 30 min (Figures 1A, 1B, and 2A; data not shown; Dustin and Springer, 1989). Additionally, both biochemical activation of MEKK2-GFP and upregulation of LFA-1 avidity occur rapidly in response to treatment with either PMA or anti-T cell receptor antibodies. Finally, after a primary TCR-mediated stimulation, both MEKK2-GFP translocation and LFA-1 avidity upregulation can recur if an activation signal is delivered a second time (data not shown; Dustin and Springer, 1989). Thus, MEKK2 is likely to be involved in delivering the TCR-mediated signals that directly or indirectly result in increased avidity of T cell LFA-1 for its ligand on APCs.

To test this hypothesis, we measured the ability of wild-type D10 cells and their MEKK2-GFP or their dnMEKK2-GFP infectants to form conjugates with antigen-pulsed APC in the absence or presence of anti-LFA-1 antibodies. Wild-type D10 cells were labeled with the green fluorescent dye CFSE (Molecular Probes), and the APC were labeled with the red fluorescent dye PKH-26 (Sigma). T cells and B cells were mixed in equal numbers and incubated for 16 min. Conjugates were counted by measuring the numbers of cell aggregates that fluoresced both green and red. As shown in Figure 4B, many such conjugates were formed when D10/MEKK2-GFP cells were incubated with conalbumin-pulsed APC. Formation of these conjugates was almost entirely dependent on the presence of antigen, since very few conjugates appeared in mixtures of D10/MEKK2-GFP cells and APC that had not been pulsed with antigen. Conjugate formation was also dependent to a large extent on LFA-1 activity, since anti-LFA-1 antibody reduced the numbers of conjugates substantially, from 21.7% to 3.5% of the cells (Figure 4B).

When these methods were applied in an antigen titration experiment, it was clear that dnMEKK2-GFP affected TCR regulation of adhesion. Wild-type D10 cells and D10/MEKK2-GFP T cells formed substantial numbers of con-
jugates at all antigen doses tested, whereas cells expressing dnMEKK2-GFP formed significantly fewer conjugates when incubated with antigen-pulsed APC, particularly as the antigen concentration was decreased (Figure 4C). Significantly, at all but the lowest dose of antigen, expression of MEKK2-GFP increased the number of conjugates formed, demonstrating that overexpression of enzymatically active MEKK2 enhances conjugate formation.

To investigate the mechanism of dnMEKK2-GFP inhibition of adhesion, conjugate assays were performed on the D10 cell lines following preincubation with anti-LFA-1 or anti-CD4 blocking antibodies. In general, D10, D10/MEKK2-GFP, and D10/dnMEKK2-GFP formed substantially fewer conjugates when preincubated with either antibody (Figure 4D). These observations demonstrate that although conjugate formation is strongly inhibited by dnMEKK2-GFP, there is not a complete block in either LFA-1-mediated adhesion or in CD4-mediated signaling events that participate in antigen-regulated adhesion.

PI3-Kinase Is a Probable Upstream Mediator of TCR Activation of MEKK2

Previous studies have shown that phosphatidylinositol 3-kinase (PI3-K) is involved in antigen-regulated T cell adhesion. Evidence from several groups indicates that PI3-K can increase LFA-1 avidity by activating cytoskeletal modifying proteins that facilitate cell spreading and membrane ruffling (Pardi et al., 1992; Shimizu and Hunt, 1996; Stewart and Hogg, 1996; Shi et al., 1997; Han et al., 1998). To determine whether PI3-K may be upstream of the MEKK2-driven regulation of T cell avidity, we studied the effects of the PI3-K inhibitor wortmannin on the binding of D10 and its derivatives to antigen-pulsed APC (Figure 5A). Consistent with the findings in Figure 4C, D10/dnMEKK2-GFP cells made fewer conjugates than either D10 or D10/MEKK2-GFP T cells. Wortmannin inhibited conjugate formation by all three cell types, and the amount of inhibition was comparable to that observed with the anti-LFA-1 antibody, consistent with the model that PI3-K is upstream of LFA-1. Additionally, the binding of anti-TCR-stimulated D10 T cells to ICAM-1 expressing fibroblasts was inhibited almost completely by wortmannin, demonstrating that TCR activation of LFA-1-mediated adhesion requires PI3-K activity (data not shown). A combination of wortmannin and anti-LFA-1 did not inhibit conjugate formation by D10 cells much more potently than either agent alone, demonstrating that the majority of the effect of PI3-K on conjugate formation is via LFA-1 (data not shown). Finally, the observation that inhibition of conjugate formation by dnMEKK2-GFP dramatically reduces the ability of wortmannin to block adhesion (Figure 5A) suggests that MEKK2 and PI3-K may be in a common pathway that connects the TCR to a cellular function that contributes to LFA-1 avidity.

In other studies, we have found that wortmannin inhibits MEKK2 activation in response to EGF or high-affinity IgE receptor ligation in T47D breast carcinoma and mast cells, respectively, demonstrating that MEKK2 activation is dependent upon PI3-K (G. J. et al., unpublished data). Figure 5B shows that TCR activation of ERK and p38 is also inhibited by wortmannin in D10 cells, indicating that MEKK2 regulation of ERK and p38 is downstream of PI3-K. Thus, several lines of evidence indicate that MEKK2 is a PI3-kinase-dependent transducer of TCR signals that activate T cell adhesion and the ERK and p38 MAPK cascades.
Discussion

We have used live-cell imaging to provide real-time analysis of the intracellular redistribution of a signaling protein in response to antigen stimulation of the T cell receptor. This study also provides evidence that MEKK2 is activated and is required for activation of the ERK and p38 MAPK pathways in response to TCR ligation. Although other MAPKKKs, particularly Raf-1, have previously been suggested to activate the ERK pathway in response to TCR stimulation (Siegel et al., 1990; Owaki et al., 1993; Siegel et al., 1993; Franklin et al., 1994; Gupta et al., 1994; Izquierdo et al., 1994), a study employing primary murine T cells has also shown that ligation of additional receptors, including CD2, CD4, and CD28, is also capable of activating Raf-1 (Siegel et al., 1993). However, ligation of CD28 does not result in p38 phosphorylation (Salmon et al., 1997). Our findings would suggest that Raf-1 is more likely to play an accessory or costimulatory role in T cell activation, whereas MEKK2 is a MAPKKK that directs antigen-regulated responses.

We have demonstrated that antigen ligation of the TCR activates MEKK2 and that dnMEKK2-GFP inhibits aspects of TCR signaling. Importantly, the effect of dnMEKK2-GFP in D10 T cells is selective for specific aspects of TCR signaling. Activation of the ERK and p38 pathways are blocked by dnMEKK2-GFP expression, but activation of JNK is not perturbed (Figures 3B–3D). Conjugate stability is inhibited, but D10/dnMEKK2-GFP cells make an amount of IL-4 essentially indistinguishable from the D10 and D10/MEKK2-GFP cell lines when activated by APC loaded with high concentrations of antigen (see Figure 4A). Because the production of IL-4 in response to TCR stimulation is a complex biological response that involves the integration of numerous signaling pathways, it seems extremely unlikely that dnMEKK2-GFP has nonspecifically perturbed signal transduction pathways. The ERK MAPK pathway has been shown to have little influence on IL-4 production (Dumont et al., 1998), consistent with our results. Additionally, we would emphasize that the kinetics of the signal transduction events inhibited by dnMEKK2-GFP (Figures 3B and 3C) are consistent with the kinetics of translocation of endogenous MEKK2 and translocation and activation of MEKK2-GFP (Figures 1A, 1B, 2A, and 3A).

Although it is reasonable to postulate that the effects of MEKK2 on T cell adhesion might be mediated by the ERK or p38 MAPK pathways, our analyses with the MEK inhibitor PD98059 and the p38 inhibitor SB203580 have shown no significant effect of these compounds on conjugate formation (data not shown). Thus MEKK2, like other MAPKKKs, including MEKK1 and PAK, appears capable of activating non-MAPK signaling pathways (Widmann et al., 1999).

As shown in Figure 5A, MEKK2 is a required signal transducer for a PI3-K-regulated adhesion pathway that contributes to increased LFA-1 avidity. Because the MEKK2 protein does not include a pleckstrin-homology domain, we postulate that TCR-mediated MEKK2 activation is downstream of one or more proteins that are directly acted upon by products of PI3-K. Given that LFA-1 avidity can be affected by diverse processes including cell spreading, LFA-1 clustering, and LFA-1 conformational changes, the mechanism(s) by which MEKK2 contributes to increased LFA-1 avidity may be quite indirect. The discovery that MEKK2 is recruited to the T cell/APC interface and regulates adhesion will allow a genetic and biochemical dissection of this TCR-regulated response, a response that has been largely refractory to analysis up to this time.

The finding that MEKK2 translocation can occur within seconds (Figures 1B and 2A) indicates that this relocalization is one of the earliest known TCR-dependent activation events (Chan et al., 1991). Interestingly, MEKK2-GFP translocation is delayed at low antigen concentration (Figures 2A and 2B), while the net percentage of total cellular MEKK2-GFP translocated to the contact site appears to be independent of antigen concentration (Figures 1D and 2A; data not shown). These observations may suggest that MEKK2 translocation reaches maximal levels only after a signaling threshold has been reached or an active signaling complex has formed. MEKK2-GFP translocation may thus be a visual proxy of TCR triggering, which occurs within seconds after the TCR has been functionally engaged. A potentially important application of our current findings would be to use recombinant MHC complexes of specific valency and geometry to define agonists that are capable of effecting TCR-mediated MEKK2-GFP translocation. Such studies may provide important information regarding the nature of the activated TCR signaling complex (Germain, 1997) and facilitate resolution of the debate concerning the minimal requirements for initiation of the TCR signaling cascade (Boniface et al., 1998; Delon et al., 1998; Vignal and Vignali, 1999).

Live cell fluorescence imaging proved to be a powerful diagnostic tool for defining subcellular relocalization and potential functions of signaling proteins during a complex biological response. This method should facilitate the characterization of other crucial signaling proteins that redistribute in response to receptor ligation or other biochemical stimuli. Beyond confirming results obtained by immunocytochemistry, live cell fluorescence imaging allows investigation of the dynamics of protein redistribution and reorganization in single cells in real time. Because this technology makes possible the visualization and dissection of rapidly occurring, complex redistribution events, live cell fluorescence imaging is a technique that makes possible the study of the temporal component of subcellular protein reorganization.

Experimental Procedures

Antibodies

The polyclonal rabbit anti-MEKK1 and anti-MEKK2 antibodies used in fluorescence microscopy experiments have been previously described (Fanger et al., 1997), and the Cy3-conjugated anti-rabbit secondary antibody was purchased from Jackson Laboratories. For some T cell/APC conjugate analyses, blocking antibodies directed against LFA-1 (I21/7.7) or CD4 (GK1.5) (Trowbridge and Omary, 1981; Dialynas et al., 1983) were used. These were prepared from hybridomas supernatants by purification over protein G agarose columns. In ELISA experiments, the monoclonal antibody BVD4-1D11 (PharMingen) ... is downstream of one or more proteins that are directly acted upon by products of PI3-K. Given that LFA-1 avidity can be affected by diverse processes including cell spreading, LFA-1 clustering, and LFA-1 conformational changes, the mechanism(s) by which MEKK2 contributes to increased LFA-1 avidity may be quite indirect. The discovery that MEKK2 is recruited to the T cell/APC interface and regulates adhesion will allow a genetic and biochemical dissection of this TCR-regulated response, a response that has been largely refractory to analysis up to this time.
Flow Cytometry Core Facility. Mixed in a 1.5 ml microcentrifuge tube with an equal volume of heparin set to 37°C. The mixture was then incubated for 30 min at 37°C to facilitate antigen–antibody interactions.

Experiments were performed with a 1:1 or 1:2 ratio of T cells to APC. For fixed cell experiments, mixing was followed by centrifugation in a Stratagene PicoFuge for 20 sec, and cell mixtures were placed in a CO2 incubator at 37°C for the indicated time periods. For immunocytochemistry experiments, T cell/APC conjugate assays were fixed with 10% formaldehyde for 15 min at room temperature, and the sample mixtures were placed in a CO2 incubator at 37°C for at least 30 min. For each sample, 20-μl cell suspensions were analyzed using a FACScan flow cytometer (Beckton-Dickinson). For each sample, 4 × 10^6 live-gated events (i.e., live cells or live cell conjugates) were collected and analyzed. Two-dimensional plots of T cells (FL1 channel, GFP and/or CFSE fluorescence) versus APC (FL2 channel, PKH-26 fluorescence) were generated. Percent conjugation analysis of the conjugate assay samples did not demonstrate a significant increase in conjugates over the 30 min preincubation period.

Cells were transferred to media lacking IL-2 approximately 20 hr before use in experiments. The CH12/LX B cell line (APC) was maintained in minimal essential media (MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum and 8 U/ml IL-2. After washing twice, cells were transferred to media lacking IL-2 approximately 20 hr before use in experiments. The CH12/LX B cell line (APC) was maintained in minimal essential media (MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum and 8 U/ml IL-2.

For fixed cell experiments, mixing was followed immediately by centrifugation in a Stratagene PicoFuge for 20 sec, and cell mixtures were placed in a CO2 incubator at 37°C for the indicated time periods. For immunocytochemistry experiments, T cell/APC conjugates were fixed with 10% formaldehyde for 15 min at room temperature, and the sample mixtures were placed in a CO2 incubator at 37°C for at least 30 min.

For each sample, 20-μl cell suspensions were analyzed using a FACScan flow cytometer (Beckton-Dickinson). For each sample, 4 × 10^6 live-gated events (i.e., live cells or live cell conjugates) were collected and analyzed. Two-dimensional plots of T cells (FL1 channel, GFP and/or CFSE fluorescence) versus APC (FL2 channel, PKH-26 fluorescence) were generated. Percent conjugation analysis of the conjugate assay samples did not demonstrate a significant increase in conjugates over the 30 min preincubation period.

Cells were transferred to media lacking IL-2 approximately 20 hr before use in experiments. The CH12/LX B cell line (APC) was maintained in minimal essential media (MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum and 8 U/ml IL-2. After washing twice, cells were transferred to media lacking IL-2 approximately 20 hr before use in experiments. The CH12/LX B cell line (APC) was maintained in minimal essential media (MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum and 8 U/ml IL-2.
For the c-jun NH₂-terminal kinase (jNK) assay, D10 T cells or D10/dnMEKK2-GFP T cells (10⁷) were mock treated or activated for 5 min at 37°C by the addition of 10 ng/ml PMA (Sigma) and 1 μM ionomycin (Calbiochem). Cells were disrupted in lysis buffer, jNK was precipitated from lysates using GST-c-jun-sepharose beads, and a GST-c-jun kinase assay was performed as previously described (Fanger et al., 1997). Reaction products were separated on a 10% SDS-PAGE gel, and 32P-labeled GST-c-jun was quantified using a phosphorimager.

**Western Blotting**

D10 T cells or D10/dnMEKK2-GFP T cells (10⁷) were pretreated as indicated in the figure legends and were then incubated for the indicated times at 37°C without stimulation or with the D10-specific TCR-activating monoclonal antibody 3D10 (10 μg/ml of Protein G-purified antibody). Protein extracts were prepared by disrupting cells in lysis buffer followed by pelleting of the nuclei by centrifugation at 12,000 x g for 10 min at 4°C. Proteins were denatured by boiling in Laemmli buffer, and 2.5 x 10⁶ cells equivalents per sample were separated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose and detected using the indicated anti-phospho-MAP kinase antibody and a standard chemiluminescence protocol. Where indicated, blots were stripped and reblotted.

**Acknowledgments**

The authors thank M. Sadelain and R. Mulligan for providing the MFG retrovirus and sequence information, G. Nolan for the Phoenix Eco retroviral packaging line, C. Janeway for the 3D3 antibody, A. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H., and Allison, K. Teague for review of the manuscript. This work was supported by grants AI-18785, AI-17134, AI-22295, DK-37875, and GM-30324.

Received February 12, 1999; revised August 10, 1999.

**References**


Live Cell Fluorescence Imaging of MEKK2


