Serial Triggering of T Cell Receptors Results in Incremental Accumulation of Signaling Intermediates*

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Triggering of the T cell receptor (TCR) leads to the production of intracellular intermediates with half-life of a few minutes. Signaling kinetics of events originating from serial TCR triggering and its relation to antigen dose was investigated. In this study we documented incremental accumulation of short-lived intermediates of the extracellular signal-regulated kinase (ERK) family, produced during successive TCR triggering. The rate and extent of the intermediate accumulation are essentially determined by the level of TCR engagement and are augmented by costimulation. ERK-1 and ERK-2 exhibit different rates of accumulation following serial receptor triggering. The data indicate that the quantitative kinetic differences in downstream signaling pathways induce qualitatively distinct biological outcomes. Although CD69, interleukin-2, and interferon-γ (IFN-γ) were primarily produced by high antigen doses that supported high MAPK phosphorylation, maximal interleukin-5 expression is induced by low and intermediate stimulus doses that do not support significant accumulation of activated ERK. We further demonstrated that the rate of phosphorylated ERK accumulation correlates with the duration of delay between T cell stimulation and the onset of IFN-γ response, with stronger stimuli giving a more rapid IFN-γ response. This delay might reflect the time required for the accumulation of signaling intermediates up to a threshold level that is necessary for activation. Thus, the data suggest that signaling events originating from serially triggered TCR are not simply sustained but are gradually accumulated and are integrated in a corresponding response.

A single peptide antigen-major histocompatibility complex (MHC) complex on the surface of an antigen-presenting cell (APC) can serially engage and trigger up to ~200 T cell receptors (TCRs) on a responding T cell (1). As a consequence of this “antigenic efficiency,” relatively small numbers of peptide-MHC complexes on APC surfaces can trigger the threshold numbers of TCRs that are required for T cell activation (2).

This intriguing feature of the T cell activation process makes physiological sense, in face of the antigenic complexity, as well as the consequent low frequency of any particular antigen, on the surface of any given APC in vivo.

Any explanation of how serially receptor triggering leads to T cell activation must reconcile two opposing kinetic features. On the one hand, the signal emanating from each triggered TCR is short-lived and, in the absence of a continuous TCR triggering, the signal will stop. This point has been established by the demonstration that interference with continuous TCR engagement by peptide-MHC complexes can terminate signaling within minutes (3, 4). Furthermore, productive TCR engagement results in rapid TCR internalization and degradation (reviewed in Ref. 5). However, on the other hand, prolonged intracellular signaling over several hours is required for T cell activation (6).

The intracellular signaling events underlying the serial triggering phenomenon are of considerable interest. The binding of TCR to a specific peptide-MHC complex initiates intracellular signaling cascade in the T cell (reviewed in Ref. 7). The earliest known step in this cascade is the induction of protein-tyrosine kinase activity, followed by downstream events, which include, among others, activation of members of the mitogen-activated protein kinase (MAPK) families (reviewed in Ref. 8) and the increase in the level of intracellular calcium. Experiments that followed calcium response in individual cells using calcium imaging have revealed that there is a delay between the TCR triggering (T cell-APC or antibody-coated bead contact) and the onset of the calcium signal. The duration of this delay decreased with increasing anti-CD3 mAb density on beads or with increasing stimuli strength (i.e. different combinations of agonist and antagonist ligands) (9, 10). These findings have led to the suggestion that the delay in calcium and other T cell responses are a consequence of the requirement for the accumulation of a threshold level of intracellular signaling complexes or events with a half-life of a few minutes (9). Thus, a pivotal aspect of T cell activation by serially triggered receptors is the accumulation of transient signaling events over the time required for activation.

Studies on the mechanisms of serial TCR triggering and sustained signaling have for the most part been performed on T cells stimulated with high, saturating stimuli doses. Although informative, such studies failed to follow the level of signaling intermediates at the initial steps of T cell activation. In the present study, we have explored the kinetics of TCR-induced signaling events and their relationships to functional responses using human T cells stimulated by a wide range of stimuli doses. We primarily followed the activation of the MAPK cascade, which consists of the extracellular signal-regulated kinases 1 and 2 (ERK-1 and ERK-2), and demonstrated that TCR-evoked signaling events are gradually accumulating rather than being sustained over the period of time following T cell stimulation.
were collected. IL-2, IL-5, and IFN-γ levels in the conditioned media were assayed by ELISA (R&D Systems, Minneapolis, MN).

RESULTS

Progressive Stimulation of MAPK Phosphorylation by Immobilized Anti-CD3, but Not by Soluble Anti-CD3 mAb—To fully activate T cells, antigen-stimulated TCR signaling needs to be sustained for up to several hours (6). As the signaling evoked by individual TCR is short-lived, sustained signaling results from ongoing TCR triggering. Surface-attached antibodies were shown to be more effective than soluble Ab in sustaining signaling (15, 16). For example, for sustained tyrosine phosphorylation and MAPK activation, an immobilized stimulus is required, whereas stimulation with soluble cross-linked anti-CD3 results in only a transient response (16).

We first compared the kinetics of TCR down-modulation induced by the two-stimulation method. Toward this end, Jurkat cells were stimulated either with soluble or with immobilized OKT3 for varying times and were then assessed for receptor down-modulation by indirect CD3 immunofluorescence and flow cytometry analysis. Stimulation of Jurkat cells with immobilized Ab induced a prolonged TCR-down-modulation that lasted up to 1 h (Fig. 1A, upper panel). The extent and frequency of TCR down-modulation correlated with the concentration of the immobilized anti-CD3 used. Stimulation with soluble anti-CD3 mAbs, on the other hand, resulted in TCR down-modulation that lasted only 15 min following stimulation, with no further receptor down-modulation at later times (Fig. 1A, lower panel).

Actin cytoskeleton was shown to be the major motor for sustained signaling that results from prolonged TCR triggering (3). Actin cytoskeleton disruption by the drug cytochalasin E inhibits signaling induced by immobilized Ab but not by cross-linked soluble anti-CD3 stimulus (16). In our hands, 4 μg cytochalasin E abolished TCR down-modulation induced by immobilized antibodies (data not shown).

These observations are consistent with the idea that, upon incubation with soluble Ab, there is a uniform distribution of Ab on the cell surface with most receptors engaged at once, and this therefore does not enable continuous receptor triggering and sustained signaling. On the other hand, stimulation with immobilized Ab provides conditions that favor receptor redistribution and serial association of TCRs with the surface-bound antibody, which leads to prolonged TCR engagements and sustained signaling.

To study the kinetics of early signaling intermediates, Jurkat cells were stimulated with various concentrations of soluble anti-CD3 mAbs, and whole cell lysates were separated on SDS-PAGE and immunoblotted with anti-phosphotyrosine and with anti-CD3 mAbs. Following stimulation with soluble cross-linked anti-CD3 (1000 ng/ml), a transient phosphorylation and tyrosine phosphorylation were evident between 1 and 15 min, which gradually declined thereafter (Fig. 1B, upper panel). Comparable kinetics of tyrosine phosphorylation were observed when cells were treated with 100 and 10 ng/ml anti-CD3 mAb (data not shown).

The same membranes were re-probed with a specific Ab for the anti-phosphorylated MAPK family members, ERK-1 and ERK-2 (Fig. 1B, lower panel). Interestingly, although MAPK phosphorylation is a relatively distal event in the TCR signaling cascade, its activation followed a time course similar to that for total tyrosine phosphorylation (Fig. 1B, lower panel). This is in agreement with the results previously shown by Berg et al. (16). As can be seen in Fig. 1B (lower panel), increasing the soluble anti-CD3 concentration from 10 to 1000 ng/ml produced higher levels of phosphorylated MAPK. However, even a 100-fold increase in anti-CD3 concentration did not significantly change the time course of the signal, which
peaked at the same time interval at all concentrations and declined thereafter.

In parallel experiments, plate-bound antibodies were used as stimulators (Fig. 1C). As was seen with soluble Ab stimulation (Fig. 1B), the maximal level of MAPK phosphorylation increased with increasing the dose of anti-CD3, and this was most pronounced when antibody dose was raised from 100 to 1000 ng/ml. However, in contrast to soluble Ab, alterations in antibody concentrations resulted in marked differences in the signaling kinetics. More specifically, MAPK phosphorylation levels rapidly increased during the initial period of time following stimulation with high anti-CD3 dose (1000 ng/ml), reaching its maximum by 7.5–15 min, and was sustained for ~30 min. The decrease seen at a later time point (60 min) is probably the result of inactivation and degradation of kinases, such as Lck, following extensive and persistent TCR triggering (17, 18).

Significantly, the level of MAPK phosphorylation gradually increased during the 60 min tested when 10–100 ng/ml anti-CD3 were used, and the response latency was inversely correlated to the anti-CD3 mAb dose. MAPK activation was hardly detected at all time points in cells stimulated with 1 ng/ml plate-bound anti-CD3 (data not shown).

To test whether the results shown above present a general phenomenon, subsequent experiments employed peripheral blood CD4+ T cells in place of Jurkat cells as responders. Stimulation of CD4+ T cells with soluble anti-CD3 resulted in a rapid accumulation of MAPK activation within 15 min, which declines thereafter (Fig. 1D, upper panel). On the other hand, when CD4+ T cells were stimulated with plate-bound anti-CD3 mAbs, gradual accumulation of signaling events was observed (Fig. 1D, lower panel). The detection of MAPK activation in CD4+ T cells stimulated by soluble or plate bound anti-CD3...
required significantly higher concentrations of Ab as compared with Jurkat cells.

The results demonstrate that cells triggered by immobilized antibodies gradually accumulate signaling events over time in an antibody dose-dependent manner and in correlation with the frequency of TCR occupancy. These findings are compatible with the notion that a minimum rate of TCR triggering is required for the accumulation of sufficient concentrations of signaling events.

Kinetics of MAPK Phosphorylation in T Cells Stimulated with Superantigens—The high affinity binding between Ab and the TCR-CD3 complex makes antibodies less efficient for serial TCR triggering compared with the peptide-MHC complex, which binds the TCR with low affinity, thus allowing dissociation following triggering. On the other hand, TCR recognition by superantigens mimics more closely that of peptide-MHC complex, as evident by a similar kinetic pattern of activation (19, 20). This is most likely because of their activity being dependent on MHC binding and to the rapid dissociation of the superantigen-TCR complex, which leads to high frequency of serial TCR triggering. We therefore followed the kinetics of MAPK activation in T cells stimulated with superantigens, which were presented by autologous monocytes, as accessory cells. It is known that interaction of ligands on the accessory cell with other T cell receptors such as the B7-CD28 interaction contribute to T cell activation. Furthermore, it was previously shown that freshly isolated monocytes have a low level of cell surface B7 expression and hence very little costimulatory activity, whereas monocytes incubated for 24 h at 37 °C provide good costimulation, which correlates with higher levels of B7 and ICAM-1 expression on their surface (Fig. 2A and Refs. 13 and 21). Most importantly, these incubation conditions have little effect on MHC class II expression, in contrast to the commonly used IFN-γ treatment for accessory cell stimulation (21). To study the kinetics of TCR-induced intracellular signaling, and the contribution of accessory receptors to this process, we compared MAPK activation in T cells stimulated with various concentrations of the superantigen SEB presented by either freshly isolated or pre-incubated monocytes (Fig. 2, B and C). T cells stimulated with pre-incubated monocytes (Fig. 2C) had considerably higher levels of MAPK phosphorylation compared with T cells stimulated with freshly isolated monocytes loaded with the same concentrations of SEB (Fig. 2B). Significantly, both the latency of the response and the time required to reach a maximal signal in each SEB concentration were greatly reduced in T cells stimulated with the pre-incubated monocytes. T cells incubated separately or with unloaded monocytes were used as controls and demonstrated insignificant levels of MAPK phosphorylation (Fig. 2E).

Various SEs such as SEB and SEA, through interaction with different T cell receptor Vβ chain, can activate distinct populations of T cells. We compared the ability of superantigens SEB and SEA to activate MAPK, using Western blotting analysis as described above. We found that both SEB and SEA induced MAPK phosphorylation, which gradually accumulated over time, reaching approximately the same maximal levels (Fig. 2, C and D). However, SEA induced significant levels of MAPK phosphorylation at earlier time points, compared with stimulation with SEB, and required considerably lower concentrations. These differences in the effective stimulatory concentrations of SEA and SEB might reflect their respective affinities for the MHC class II molecules (22). However, in the present study, the superantigens were loaded onto the monocytes at 4 °C; at this temperature, no significant differences in the binding affinities of the two superantigens to class II were observed (22). It is possible, therefore, that other factors contribute to the observed differences between SEA and SEB activity. One such factor could be differences in the affinities of the two SEs to the TCR, which lead to different frequencies of receptor engagement. In fact, several studies have indicated a relationship between the overall extent of TCR down-modulation and ligand potency (1, 23–25).

To establish the extent of TCR occupancy achieved by the two superantigens, T cells were incubated with APCs bearing various doses of either SEA or SEB and then stained for surface TCR-Vβ5 or -Vβ17, respectively. The maximal level of TCR down-modulation was similar in both SEA- and SEB-stimulated cells. However, in agreement with expectations, SEA was much better in inducing TCR down-modulation as compared with SEB, at the lower antigen dosage (Fig. 2F). Although no Vβ17 down-modulation was induced by 0.1–1 ng/ml SEB, a total of 10–20% of Vβ5 TCR are down-modulated when cells are stimulated with the same concentrations of SEA.

Thus, the level of MAPK phosphorylation induced by serially triggered TCRs increased over a relatively long period of time, and the rate of this increase correlated with the extent of TCR engagement as measured by receptor down-modulation. Both the extent of TCR engagement and the corresponding signaling depended on the nature of the antigen and its dose. Furthermore, costimulation amplified receptor signaling and facilitated its accumulation in the face of a fixed antigen dose.

Differential Effects of Antigen Dose on CD69, IL-2, IFN-γ, and IL-5 Production—In light of the results presented above, we hypothesized that the quantitative differences in strength and duration of downstream signaling pathways can induce qualitatively distinct biological outcomes. Dumont et al. (26, 27) have previously demonstrated that distinct signaling pathways contribute differently to the regulation of cytokine response in T cells. The authors have disrupted ERK-1/2 activation by using PD98059, a selective inhibitor of MAP kinase 1 (26, 27). Treatment of stimulated T cells with PD98059 resulted in marked inhibition of CD69, IL-2, and IFN-γ expression. However, even at high concentrations, PD98059 not only failed to suppress IL-4 and IL-5, but it significantly augmented their production (12).

In view of ERK’s unique role in regulating distinct T cell responses, we sought to correlate the rate of accumulation of activated ERK, resulting from increasing doses of the SEB and SEA stimulators, with the level of production of individual cytokines. T cells were combined with monocytes that were preloaded with various concentrations of either SEB or SEA. After 48 h of culture, the level of CD69 expression on the cell surface was measured by flow cytometric analysis, and the level of cytokines secreted to the medium was determined by ELISA.

The results revealed that CD69 expression by individual cells as well as IFN-γ and IL-2 secretion to the culture media increased with increasing SEB concentrations. IL-5 secretion, on the other hand, peaked at 100-fold lower SEB concentration (10 ng/ml) and decreased at higher doses (Fig. 3). Giving the differential role ERK activation plays in the expression of these proteins, their levels of expression correlated with the extent of phosphorylated ERK accumulation at each antigen dose. Likewise, SEA with its capacity to induce rapid ERK-1/2 activation at lower stimuli doses, as compared with SEB (i.e. 0.1–10 ng/ml), induced significant levels of CD69 and IFN-γ at these doses, whereas only marginal levels of these proteins were detected in SEB-stimulated cells (Fig. 3). The levels of IL-5 were relatively low when SEA was used as stimulator at all doses except for a peak at 0.1 ng/ml, consistent with its ability to induce significant ERK phosphorylation even at the intermediate dosage range. Of note, the levels of IL-4 detected in the
FIG. 2. Kinetics of MAPK phosphorylation and TCR down-modulation following superantigen stimulation. A, expression of cell surface antigens on resting and activated monocytes. Purified resting monocytes or monocytes that were “pre-activated” by incubation in medium at 37 °C were stained with anti-CD86, CTLA-4Ig, anti-CD54, and anti-MHC class II (solid lines). The dashed lines represent staining with control reagents. CD4+ T cells (2.5 × 10^6) were conjugated with either freshly isolated (B) or pre-activated (C–F) autologous monocytes (2.5 × 10^6) that were pulsed with the indicated concentrations of the superantigens SEB (B and C) or SEA (D). The conjugates were then incubated at 37 °C for the indicated times. E, negative controls: T cells incubated for 120 min in the absence of monocytes (lane 1), monocytes loaded with 1000 ng/ml SEB incubated for 120 min in the absence of T cells (lane 2), T cells that were conjugated with unpulsed monocytes and incubated for 120 min (lane 3), and T cells and monocytes loaded with 1000 ng/ml SEB at time 0 (lane 4). Dually phosphorylated MAPK was detected in detergent lysates of these cells after Western blotting as described in Fig. 1. Immunoblotting for Vav confirmed comparable loading of protein in each lane as in Fig. 1C. F, CD4+ T cells and either SEA- or SEB-pulsed monocytes were allowed to form conjugates as described above and were then incubated at 37 °C for 16 h. The level of TCRβ3 or TCRγδ17 expression in SEA and SEB stimulated cells, respectively, was determined using the appropriate anti-TCR antibodies and fluorescence-activated cell sorting analysis. The data are presented as percentage of TCR down-modulation. 100% expression is the level of TCR expression on T cells cultured with unpulsed APCs. The data are from one experiment; similar results were obtained in two other experiments.
culture supernatants under the conditions used in these experiments were too low to permit reliable measurements.

In aggregate, there is a correlation between the extent of ERK activation (seen in Fig. 2) and the induction of ERK-dependent T cell responses (i.e., CD69, IFN-γ, and IL-2) by each antigen and its respective dose. In contrast, maximal IL-5 expression is induced by stimulus doses that do not support significant accumulation of activated ERK. A similar correlation among antigen dose, the extent of TCR down-modulation, and the induction of effector function was described previously (24). It is especially interesting to note, in this regard, that CD69 up-regulation was elicited at low antigen concentrations whereas up to 1000-fold more antigen was required for IFN-γ secretion and proliferation.

Antigen Dose Dictates the Stimulation Time Required for IFN-γ Production—The data above demonstrated that the magnitude of ERK-dependent T cell responses, as measured by IFN-γ, IL-2, and CD69 expression, correlates with the antigen dose. These findings are in agreement with the notion that higher stimulus dose gives stronger T cell response. However, the data also demonstrated that antigen dose determines the time lag between T cell stimulation and the onset of ERK activation, and the rate of its accumulation. Therefore, it seems reasonable to assume, based on the present data, that an increased stimuli dose, which reduces the accumulation time required to reach a threshold level of signaling intermediates, will consequently shorten the time required for the cytokine response. To test this assumption, T cells were stimulated by monocytes loaded with various doses of SEB and conditioned medium was collected after different times of incubation and tested for the presence of IFN-γ (Fig. 4). Interestingly, the time required for the onset of the IFN-γ response correlated well with SEB concentration. IFN-γ production by T cells stimulated with high SEB doses (100 and 1000 ng/ml) was rapid and took place within 5 h of stimulation. A longer period of 8 and 20 h was required for IFN-γ production by cells stimulated with 10 and 1 ng/ml, respectively. Remarkably, despite the differences in the antigenic doses used, similar initial levels of IFN-γ were detected in culture media of cells stimulated with the various concentrations, although detected at different time points following stimulation, suggesting a threshold effect for IFN-γ production. This latter observation is not a consequence of the IFN-γ detection sensitivity, as these concentrations (~100 pg/ml) are significantly higher than the lower limit of detection by the ELISA used (5 pg/ml). Thus, stimulus dose augments T cell activation, at least in part, by reducing the time required for IFN-γ secretion.

DISCUSSION

Commitment of T cells to cytokine production and proliferation requires sustained TCR signaling for up to several hours, which is achieved through serial receptor engagement. In the present study, we characterized the kinetics of early signaling events during the course of serial TCR triggering. The principal experimental finding in this study is that signaling intermediates produced by serially triggered TCRs are not simply sustained but are incrementally building up. Increased signaling levels during T cell stimulation were also observed by Muller et
The data demonstrated that the onset of ERK phosphorylation and the rate of its accumulation were dependent on the antigen dose. A previous report has established that the number of TCR triggered per time correlated with stimulus dose as well as with antigen-TCR dissociation rate (29). Thus, the extent of accumulation of signaling events reflected the level of antigenic stimulation, which was determined by antigen dose and the dissociation rate of the TCR-ligand complex, with higher dissociation rate resulting in increased rate of intermediate accumulation. Differences in dissociation rate and consequently the frequency of TCR engagement can explain the differences between SEA and SEB in regard to their respective ability to induce ERK phosphorylation and TCR down-modulation.

Hudrisier et al. (30) have shown that the magnitude of the functional cytotoxic T lymphocyte response is directly related to the frequency of serial TCR engagement, which, in turn, is determined by the rate of ligand-TCR complex dissociation. Our data support these findings and provide an explanation for how the priming conditions such as the nature of the antigen and its dose are integrated in a corresponding response. Accordingly, the amplitude of the biological response is determined by the level of accumulation of signaling intermediates within the cell, which in turn are determined by the level of TCR occupancy.

Furthermore, antigen dose and the frequency of TCR engagement dictate not only the amplitude of the response, but also the rate of accumulation of signaling intermediates and as a result the duration of signaling required to reach threshold level. In accord with the above proposition, we demonstrated that the stimulation time required for IFN-γ production inversely correlated with antigenic dose, with higher doses giving a more rapid response. The results also demonstrated that although they emeranated after different times following stimulation, the initial levels of IFN-γ induced by low and high antigen concentrations were similar, suggesting a threshold effect. We suggest that the frequency of TCR engagement, which depends on antigen nature and dose, dictates the accumulation rate of triggered events and, hence, the time required to reach threshold levels and activation.

Besides the level of TCR triggering, we demonstrated that costimulation also plays an important role in determining the extent and rate of intermediate accumulation. In this regard, it was shown that the role of costimulation is not to increase the number of triggered TCRs, but rather to contribute to TCR signaling by stabilizing tyrosine phosphorylation (18); in this way, costimulation reduces the number of triggered TCR required to reach activation threshold. Taken together, costimulation is thus predicted to shorten signaling time leading to a biological response. Indeed, it was previously shown that the time required for commitment of T cell to proliferation can be shortened by providing costimulation and by increasing antigen dose (31). Significantly, in this report, T cells stimulated with high or low doses of anti-CD3 in combination with anti-CD28 required short and long stimulation time for commitment, respectively, but ultimately reached the same proliferative response. Thus, both the antigenic dose and the presence of costimulation determine the duration of signaling required for T cell activation.

Together, these observations are consistent with the model proposed by Wulfing et al. (9), to explain the correlation between the duration of delay in the onset of calcium response and the strength of the stimulus. Accordingly, the accumulation of intracellular intermediates with a short half-life is the rate-limiting step in triggering the calcium response. Using different experimental systems, Gunzer et al. (32) have arrived to a similar conclusions. Having shown that a single T cell can sequentially encounter one or more APCs in multiple short-lived interactions, which eventually trigger T cell activation, the authors have suggested a cumulative signaling threshold based on both the frequency of interactions and their respective strength (32). The data in the present study are the first to directly demonstrate the accumulation of short-lived intracellular intermediates that are produced by successive receptor triggering, most likely, up to a threshold level required for activation of the biological response.

It was previously suggested that the level of TCR occupancy could control the amplitude of the T cell response as well as its type (1). In accord with this proposal, we documented differences in the antigenic dose required for the activation of individual responses, which correlated with the rate and extent of ERK phosphorylation. These findings are in agreement with a previous report, which has shown that distinct activation pathways contribute differently to the production of various cytokines (12). Accordingly, the ERK signaling pathway is essential for both IFN-γ and IL-2 production, which are associated with Th1 response, and inhibition of this pathway augments Th2-associated cytokines. Our findings, as well as previous reports (33, 34), imply that, whereas intermediate dose of stimuli induced T cell response that is skewed toward Th2-like cytokine (IL-5), priming with higher doses of stimuli favored increased production of Th1-like cytokines (IFN-γ, IL-2), correlating with the respective abilities of the various stimuli doses to induce ERK-phosphorylation. Fully polarized response requires prolonged TCR stimulation (31) and the presence of certain cytokines, which was not tested here. However, these results propose that the extent of antigenic stimulation biases the response toward certain Th-like phenotypes and contributes to the subsequent acquisition of distinct type of response. Furthermore, the data provide a possible mechanistic insight into this process. Thus, although Th1/Th2 development is primarily driven by cytokines (such as IL-12 and IL-4), priming conditions such as the strength of antigenic stimulation play an important role in the decision (33–35).

A recent report has revealed distinct activation thresholds for the three MAPK families: ERK, c-Jun N-terminal kinase, and p38 (11). Hence, differences in signaling strength induce selective activation of the different MAPK families. Although activation of other MAPK families was not examined in the present study (the diphosphorylated c-Jun N-terminal kinase was not detected, probably reflecting its higher activation threshold), the data in hand (Fig. 2) demonstrate higher level of ERK-2 (p42) phosphorylation and a faster onset of appearance than ERK-1 (p44). These differences in the activation levels of ERK-1 and ERK-2 are most apparent at the lower superantigen concentrations (Fig. 2, A and B), and are less prominent in cells stimulated with immobilized anti-CD3 mAbs (see Fig. 1C). One possible explanation for the asymmetries seen in the Western blot data for ERK-1 and ERK-2 lies in the characteristics of the antibodies used in the assay. It is possible that the antibodies are more sensitive detectors of the ERK-2 than the ERK-1. However, the observation that the proportion and kinetics of ERK-1 and ERK-2 activation were similar in T cells stimulated with soluble anti-CD3 mAbs (Fig. 1B, lower panel) suggests that this is not the case. Moreover, this latter observation indicates that the observed differences between ERK-1 and ERK-2 activation are attributable to distinct accumulation rates of the two ERKs that are apparent when serial receptor triggering is taking place. These accumulation rates are not dependent on their activation thresholds, as both ERKs share...
similar activation thresholds (Fig. 1B and Ref. 11). We therefore suggest that there are distinct time thresholds for the accumulation of ERK-1 and ERK-2 and possibly for other signaling pathways, which are distinct from activation thresholds. These differences in signal strength can induce quantitatively and qualitatively different signaling pathways. In this regard, Pages et al. (36) have demonstrated, using ERK-1-deficient mice, that although for most part ERK-1 was dispensable, and ERK-2 may have compensated for its loss, thymocytes stimulated by anti-TCR mAbs had severely reduced proliferative response, and thymocyte maturation was also defective in those mice. Thus, ERK-1 and ERK-2 apparently have distinct roles in thymocyte development. Furthermore, it was suggested that the two ERK isoforms compete with each other for the upstream MAPK kinase activator (36). This latter point provides a possible mechanism for our observation regarding the quantitative and qualitative aspects of the biological outcome. This mechanistic insight into the kinetics of signaling events during TCR serial triggering provides a useful framework for further exploring the intracellular pathways influenced by this process and its impact on T cell fate.

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