

Stimulus-Specific Distinctions in Spatial and Temporal Dynamics of Stress-Activated Protein Kinase Kinase Kinases Revealed by a Fluorescence Resonance Energy Transfer Biosensor[▽]

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The stress-activated protein kinases (SAPKs), namely, p38 and JNK, are members of the mitogen-activated protein kinase family and are important determinants of cell fate when cells are exposed to environmental stresses such as UV and osmotic stress. SAPKs are activated by SAPK kinases (SAP2Ks), which are in turn activated by various SAP2K kinases (SAP3Ks). Because conventional methods, such as immunoblotting using phospho-specific antibodies, measure the average activity of SAP3Ks in a cell population, the intracellular dynamics of SAP3K activity are largely unknown. Here, we developed a reporter of SAP3K activity toward the MKK6 SAP2K, based on fluorescence resonance energy transfer, that can uncover the dynamic behavior of SAP3K activation in cells. Using this reporter, we demonstrated that SAP3K activation occurs either synchronously or asynchronously among a cell population and in different cellular compartments in single cells, depending on the type of stress applied. In particular, SAP3Ks are activated by epidermal growth factor and osmotic stress on the plasma membrane, by anisomycin and UV in the cytoplasm, and by etoposide in the nucleus. These observations revealed previously unknown heterogeneity in SAPK responses and supplied answers to the question of the cellular location in which various stresses induce stimulus-specific SAPK responses.

Mitogen-activated protein kinases (MAPKs) are important intracellular signaling molecules that are activated through kinase cascades composed of a MAPK kinase (MAP2K) kinase (MAP3K), a MAP2K, and a MAPK (2). In mammalian cells, several MAPK cascades coexist: the growth-promoting extracellular signal-regulated kinase MAPK cascade and the growth suppressing stress-activated protein kinase (SAPK) cascades. Together, these kinase cascades serve important roles in making the critical choice between growth and apoptosis under various environmental conditions (25). Two families of SAPKs, namely, JNK and p38, are activated through kinase cascades composed of a SAPK kinase (SAP2K) kinase (SAP3K) and a SAP2K by a variety of environmental (physical and chemical) stresses such as UV, gamma rays, translation inhibitors, osmotic stress, and oxidative stress as well as by physiological mediators such as transforming growth factor β , interleukin-1 β (IL-1 β), and tumor necrosis factor alpha (TNF- α). The SAPK cascades are also important in embryonic development and in immune responses in the adult organism and are therapeutic targets for inflammatory diseases, cancer, and other pathological conditions (1, 3, 13). The p38 SAPK is activated mainly by the MKK3 and MKK6 SAP2Ks, whereas the JNK SAPK is activated by the MKK4 and MKK7 SAP2Ks. These SAP2Ks are in turn activated by diverse SAP3Ks including MEKK1, MEKK2, MEKK3, MTK1 (MEKK4), TAK1, ASKs, MLKs, and TAOs (2).

This large array of SAP3Ks undoubtedly reflects the diversity of the stress stimuli that can activate the SAPK cascades. In

spite of extensive investigation, however, the molecular mechanisms by which stresses such as UV-C activate SAP3Ks are still largely unknown and remain controversial. UV-C can be absorbed by, and affect the chemistry of, proteins, RNA, and DNA. Potentially, therefore, UV-C may initiate the activation of the SAPK pathway on the cell membrane, in the cytoplasm, or in the nucleus. In particular, one theory posits that UV-C radiation induces the clustering and activation of cell surface receptors for epidermal growth factor (EGF), TNF- α , and IL-1 (15, 16). According to this theory, UV-C initiates SAPK responses in the vicinity of the cell surface. An alternative proposal is that UV-C is absorbed by the ribosomes and that the stalled translation machinery then generates a signal leading to SAPK activation (5). According to this model, UV-C should initiate SAPK responses in the cytoplasm. One possible approach to distinguish these models is to identify the subcellular location where SAP3K activation primarily takes place. However, currently available methods do not allow such a measurement.

The activity of SAP3Ks has been conventionally monitored either by measurements of their kinase catalytic activities *in vitro* or, more frequently, by the detection of the phosphorylation status of their specific substrates, such as MKK3 and MKK6, by immunoblotting or immunofluorescence staining with phospho-specific antibodies. Although these methods have provided a valuable insight into MAP3K functions and regulation, they can measure only the average activity of SAP3Ks in a cell population. Furthermore, they cannot easily distinguish the different subcellular locations where SAP3K activation might take place. As a consequence, the intracellular dynamics of SAP3K activity are currently largely unknown. Here we report the development, validation, and practical application of a fluorescence resonance energy transfer (FRET)-

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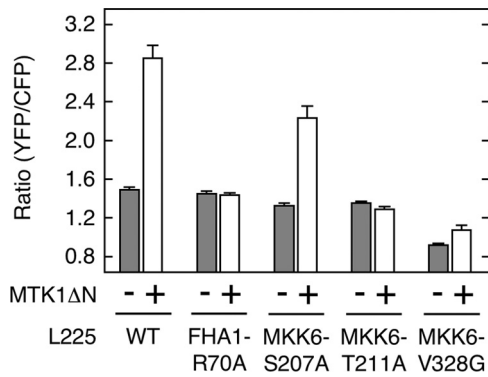


FIG. 2. Effect of various mutations in L225 on the MTK1ΔN-induced FRET change. In vivo YFP/CFP emission ratio changes (means $\pm$ SEM) of the SAP3K reporter L225, or its mutant derivatives, cotransfected without (gray) or with (white) the constitutively active MTK1ΔN kinase are shown. FHA1-R70A is a non-phosphothreonine-binding mutant of the FHA1 domain. MKK6-S207A and MKK6-T211A are nonphosphorylating mutants of the MKK6 activation loop. MKK6-V328G is a nondocking mutant of the MKK6 DVD domain. WT, wild type.

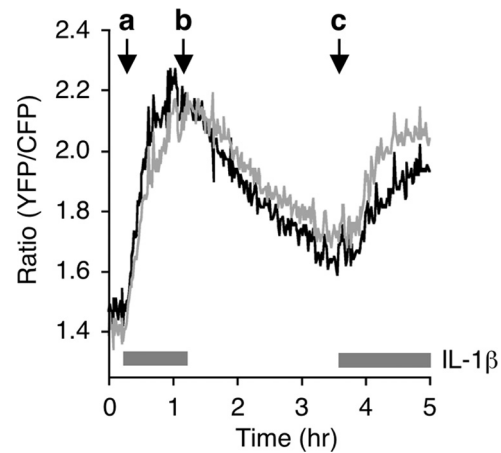


FIG. 4. Reversibility of L225 reporter activity. HeLa cells expressing the L225 reporter were stimulated with IL-1 β (10 ng/ml) at time a, washed out at time b, and restimulated with IL-1 β at time c. The time course of the YFP/CFP emission ratio was monitored for two separate cells (black and gray curves).

to 0.3 μ g) of an expression plasmid for constitutively active MTK1ΔN. The total amount of plasmid DNA was adjusted to 0.4 μ g per dish using the empty vector pcDNA3. Immediately after the fluorescent spectrum was recorded, the cells were washed twice with ice-cold phosphate-buffered saline and lysed directly in SDS sample buffer. Samples were then boiled for 5 min, separated by SDS-

polyacrylamide gel electrophoresis (10% polyacrylamide gel), transferred onto a nitrocellulose membrane, and immunoblotted with anti-phospho-MKK3/6 (Bio-source) and anti-green fluorescent protein (anti-GFP) (MBL) antibodies. The anti-GFP antibody also recognizes YFP and CFP.

HeLa cells were starved in 0.5% fetal bovine serum containing DMEM for 2 h before stimulation with either TNF- α (10 ng/ml), anisomycin (10 μ g/ml), or

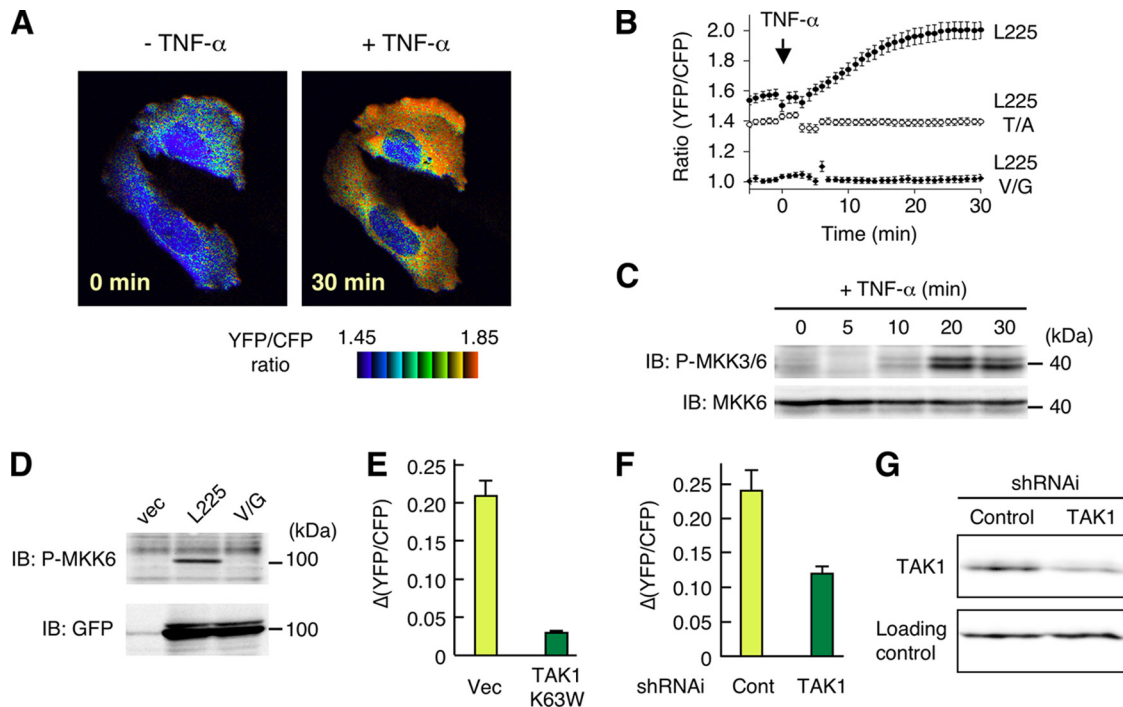


FIG. 3. Detection of SAP3K activation in individual cells. (A and B) Cell images (A) and time courses (B) of the YFP/CFP emission ratios of L225 reporters in response to TNF- α using HeLa cells. T/A, T211A; V/G, V328G. (C) Phosphorylation of endogenous MKK3/6 following stimulation of HeLa cells by TNF- α . Total cell lysates were probed either with anti-phospho-MKK3/6 antibody (top) or with anti-MKK3/6 antibody (bottom). IB, immunoblot. (D) Phosphorylation of L225 reporters following stimulation of the transfected HeLa cells by TNF- α as described above for B. Anti-GFP antibody reacts with YFP and CFP in L225 (bottom). (E and F) Expression of the dominant negative TAK1-K63W mutant (E) or TAK1 shRNAi (F) inhibits TAK1 activation by TNF- α (10 ng/ml for 30 min), as revealed by a decrease in the change in the YFP/CFP ratio [Δ (YFP/CFP)] of L225. Vec, vector; Cont, control. (G) shRNAi inhibition of TAK1 expression. Immunoblot analysis of the effect of TAK1-specific shRNAi on TAK1 protein expression is shown. The control is luciferase-specific shRNAi. A nonspecific band serves as a loading control.

methyl methanesulfonate (MMS) (100 μ g/ml). At the indicated times after stimulation, cells were rapidly washed and lysed as described above. Anti-phospho-MKK3/6 (Cell Signaling Technology) and anti-MKK3/6 (SantaCruz) antibodies were used for the detection of endogenous MKK3/6 phosphorylation and protein levels, respectively.

Dominant-inhibitory TAK1 mutant. HeLa cells were cotransfected with 0.1 μ g of the L225 reporter plasmid and 0.3 μ g of the TAK1-K63W expression plasmid. TAK1 activation by TNF- α was analyzed approximately 48 to 72 h after transfection as described above.

shRNAi knockdown. short hairpin RNA interference (shRNAi) knockdown plasmids for TAK1 and control luciferase were generated by inserting the following synthetic oligonucleotides into the pSUPERretro vector according to the manufacturer's protocol (Oligoengine) (the TAK1 shRNAi sequence is based on data from a previous report [20], and the nucleotides in capital letters form a hairpin): TAK1-1 (5'-gatccccGACTGTGACTGTAAGTGAATcaagagaTTCCAGTTACAGTCAAGTCtttta), TAK1-2 (5'-agcttaaaaaGACTGTGACTGTAAGTGAATcttgaTTCCA GTTACAGTCAAGTCggg), Luciferase-1 (5'-gatccccCGTACGCGGAATACTTC GATcaagagaTCGAAGTATTCGCGGTACGtttta), and Luciferase-2 (5'-agcttaaaaa aCGTACGCGGAATACTTCGATcttgaTCGAAGTATTCGCGGTACGggg).

HeLa cells were cotransfected with 0.05 μ g of the L225 reporter plasmid and 0.35 μ g of the shRNAi plasmid and were analyzed by fluorescent microscopy approximately 48 to 72 h after transfection. To confirm the efficiency of TAK1 silencing, HeLa cells were transfected with plasmid pSuperRetro-shRNA for 24 h and were selected with puromycin for an additional 48 h. Total cell lysates were analyzed by immunoblotting with an anti-TAK1 antibody (Sigma).

RESULTS

Construction of an efficient SAP3K reporter. The SAP3K activity reporter that we designed is composed of an N-terminal YFP followed by the phosphothreonine binding FHA1 domain of yeast Rad53, the C-terminal lobe of the kinase domain of human MKK6 including the phosphorylation sites (Ser-207 and Thr-211), and a C-terminal CFP (Fig. 1A). The phosphorylation of MKK6 by activated SAP3K will lead to FHA1 domain binding, and the subsequent closer association between YFP and CFP will enhance the FRET efficiency. The general architecture of this SAP3K reporter is similar to that of the previously successful protein kinase A, protein kinase C, and Src reporters (24, 26, 27). However, several modifications had to be made to generate an efficient reporter. To assess the performances of the various reporter constructs, we coexpressed them in COS-7 cells together with MTK1 Δ N, a constitutively active version of a SAP3K that phosphorylates MKK6 (21). The reporters were excited at 405 nm, and the ratio of the fluorescence emissions at the YFP peak (530 nm) to those at the CFP peak (480 nm) was calculated. The emission spectrum of the best reporter, L225, showed that its relative FRET ratio change (RRC) was about 69% of that of the vector control (Fig. 1B). The RRC is defined as follows: $RRC = [(YFP/CFP)_{activated} - (YFP/CFP)_{basal}] / (YFP/CFP)_{basal}$.

A brief summary of the reporter modifications is as follows. First, instead of a short substrate peptide, we included the entire C lobe of MKK6 (aa 132 to 334) that contains the DVD docking domain specific to SAP3Ks (aa 311 to 334) (8, 22). Second, the amino acid sequence around the MKK6 phosphorylation sites was optimized for FHA1 recognition by mutating SVAKTIDA (aa 207 to 214) to SVAKTIAD (4). As will be shown below, the modified sequence can be phosphorylated by SAP3Ks as efficiently as the wild-type sequence. Third, we employed brighter variants of YFP, namely, Venus or YPet, and a brighter version of CFP, namely, SECFP (10–12). Fourth, we varied the lengths and the sequences of the linkers (L1, L2, and L3). Finally, we found that deleting Pro-218 near

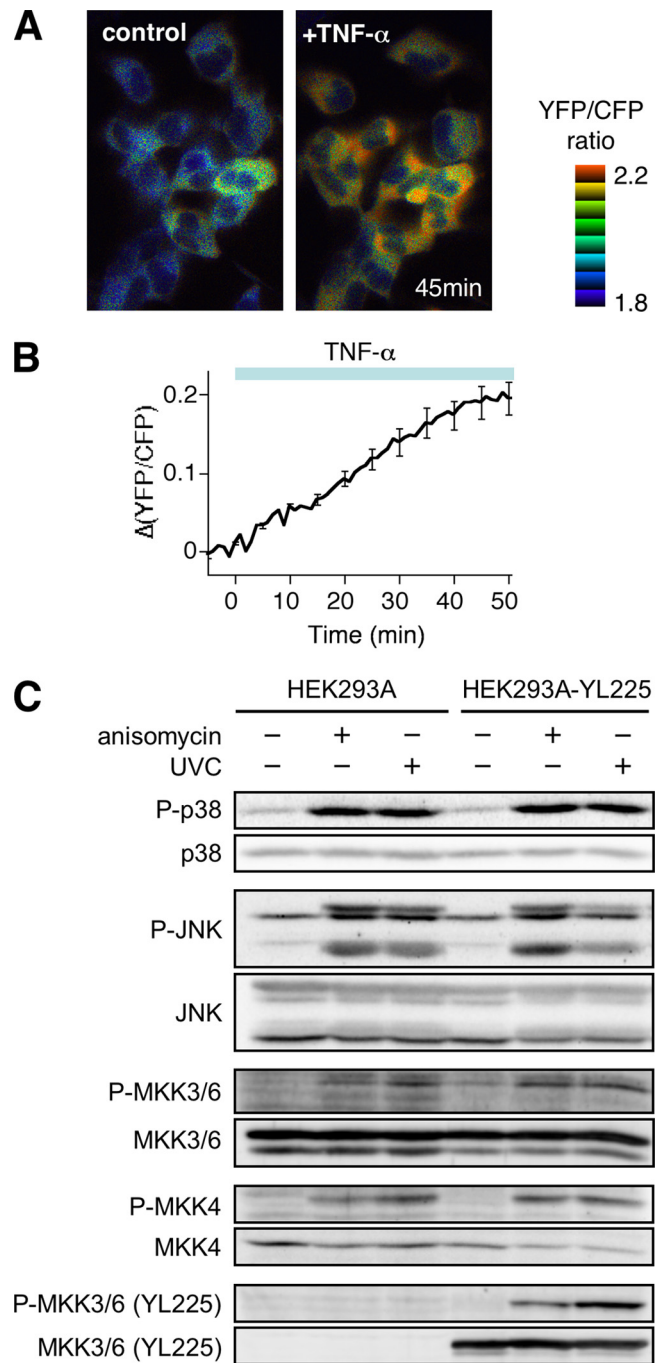


FIG. 5. Cells stably expressing YL225. (A) Images of the YFP/CFP ratio of HEK293A-YL225 cells before and after TNF- α (10 ng/ml) stimulation. (B) Time course of the change in YFP/CFP ratios of cells shown in panel A. The means $\pm$ SEM ($n = 9$) are shown. (C) Immunoblot analysis of the anisomycin (10 ng/ml)- and UV-C (80 J/m²)-induced phosphorylation of endogenous p38, JNK, MKK3, MKK6, MKK4, and the stably expressed YL225 probe.

the phosphorylation site greatly improved the FRET response. It is likely that the elimination of the proline residue allowed more flexible interactions between phosphorylated Thr-211 and the FHA1 domain.

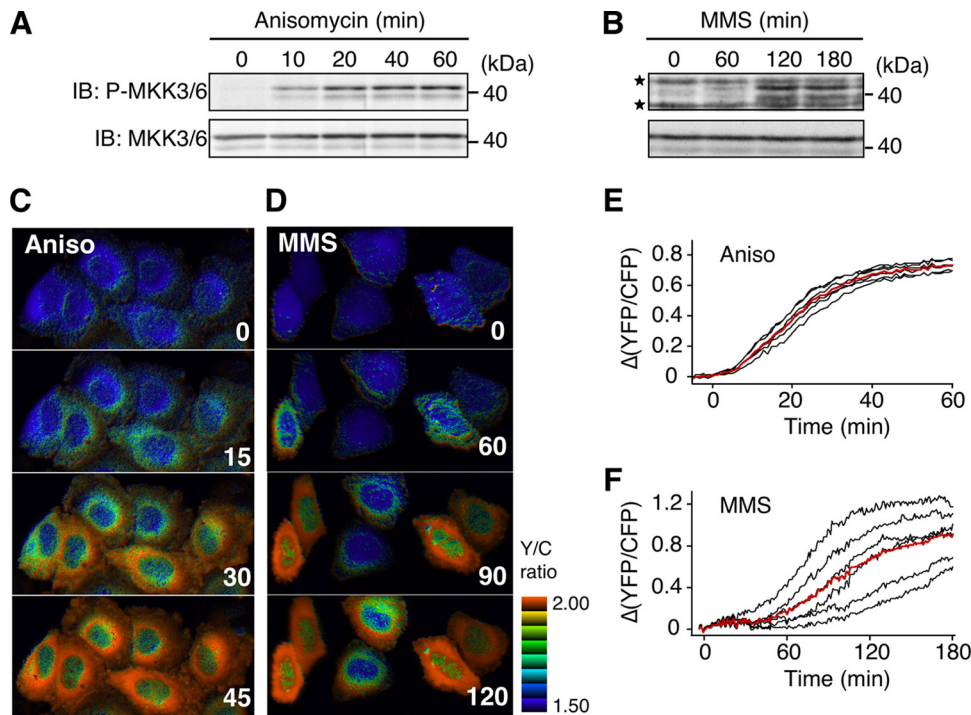


FIG. 6. Temporal dynamics of SAP3K activation in single cells. (A and B) Immunoblot (IB) analyses of MKK3/6 phosphorylation in HeLa cells following stimulation with anisomycin (A) or MMS (B). The stars indicate nonspecific bands. (C and D) Cell images of the L225 YFP/CFP emission ratios in response to anisomycin (Aniso) (C) or to MMS (D). HeLa cells were transfected with the L225 expression plasmid. Numbers are minutes after stimulation. Three different fields from one picture were spliced together to save space (D). (E and F) Time course of the changes in the YFP/CFP ratio in HeLa cells stimulated with either anisomycin (E) or MMS (F). Black curves represent the six cells shown in panels C and D, respectively, while the red curves represent the average values.

Selectivity and quantitative accuracy of the SAP3K reporter.

Selectivity and quantitative accuracy are the two important factors for a kinase activity reporter. The coexpression of the reporter in cells with the active forms of SAP3Ks such as MTK1 Δ N, MEKK1 Δ N, or ASK1 increased the YFP/CFP emission ratio of L225 (Fig. 1C). In contrast, the constitutively active form of Raf-1 (Raf-1 Δ N), which does not activate MKK6, was ineffective. The expression of full-length MTK1 or TAK1, which are inactive in the absence of their respective activators (6, 9), did not increase the FRET efficiency. However, when these SAP3Ks were coexpressed with their respective activators, GADD45 β and TAB1 (18, 21), large increases in FRET efficiency were observed (Fig. 1C). When increasing amounts of MTK1 Δ N were expressed in COS-7 cells, the YFP/CFP ratio of L225 increased with a dose dependence similar to that for L225 phosphorylation assessed by immunoblotting (Fig. 1D and E). Thus, the L225 reporter selectively and accurately reports the status of SAP3K activity in cells.

To verify that L225 functions according to our design, we individually mutated the FHA1 domain, the MKK6 phosphorylation sites, and the DVD docking site. A non-phospho-Thr-binding mutation of FHA1 (corresponding to Rad53 R70A) or an Ala substitution of the MKK6 phosphorylation site, Thr-211, completely abolished the FRET change induced by MTK1 Δ N (Fig. 2). In contrast, an Ala substitution of Ser-207, which is not a part of the FHA1 recognition motif, had no effect. A mutation in the DVD domain (corresponding to MKK6-V328G) resulted in a lower basal YFP/CFP ratio and

an inhibition of the MTK1 Δ N-induced YFP/CFP ratio increase. Thus, these results indicate that L225 reports the status of SAP3K activity according to our original design.

Monitoring of SAP3K activity in living cells. To test if L225 can detect physiologically induced SAP3K activity in live cells, we stimulated L225-expressing HeLa cells with TNF- α (19). TNF- α rapidly induced an increase in the YFP/CFP ratio by simultaneously increasing YFP fluorescence and decreasing CFP fluorescence (data not shown). Typical images are shown in Fig. 3A. The time course of the increase in the YFP/CFP ratio (Fig. 3B) was comparable to that of the phosphorylation of endogenous MKK3 and MKK6 (Fig. 3C). Neither the DVD mutant V328G (V/G) nor the phosphorylation site mutant T211A (T/A) of L225 responded to TNF- α stimulation (Fig. 3B), consistent with the fact that TNF- α stimulation did not induce any phosphorylation of L225 V/G (Fig. 3D). In agreement with a previously reported observation that TAK1 is essential for SAPK activation by TNF- α (17), the FRET increase induced by TNF- α was significantly weaker when endogenous TAK1 kinase activity was inhibited by dominant negative TAK1-K63W (Fig. 3E) or by the expression of shRNAi specific for TAK1 (Fig. 3F and G).

We also tested the reversibility of the L225 reporter. When cells were challenged with IL-1 β , another cytokine that activates TAK1, the YFP/CFP ratio increased (Fig. 4). When the cytokine was washed out, the YFP/CFP ratio decreased toward the prestimulation level, indicating that the L225 reporter is subject to dephosphorylation by endogenous phosphatases.

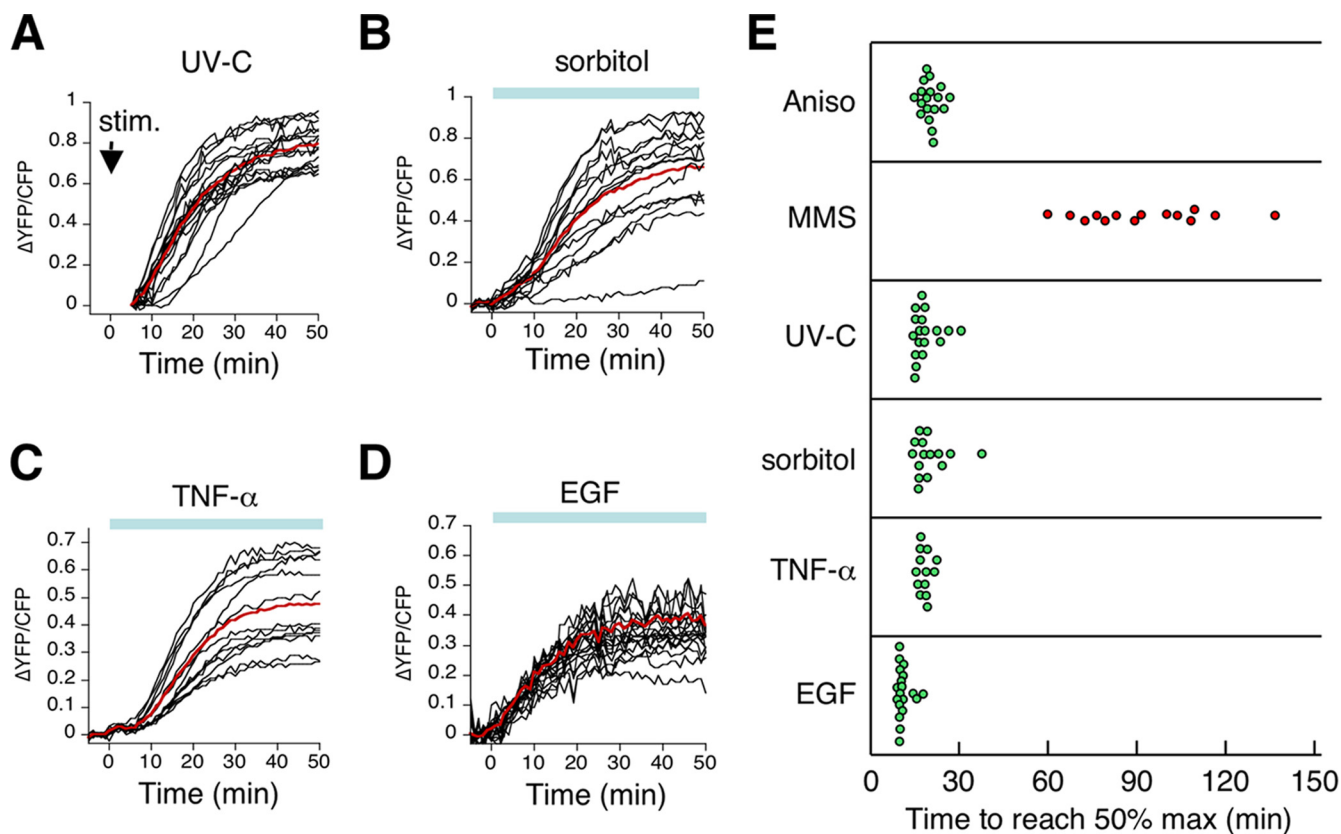


FIG. 7. Temporal dynamics of SAP3K activation by various stimuli in single cells. (A to D) Time course of the changes in the YFP/CFP ratio ($\Delta YFP/CFP$) in individual HeLa cells expressing L225 (A to C) or YL225-pm (D) following stimulation (stim.) with either 80 J/m² UV-C irradiation (A), 0.4 M sorbitol (B), 10 ng/ml TNF- α (C), or 100 ng/ml EGF (D). Average traces are shown in red. For UV-C, image acquisition was started at 5 min after UV irradiation. (E) Distribution of the time required to reach 50% of the maximum $\Delta YFP/CFP$ increase in individual cells, calculated from the data sets shown in panels A to D and in Fig. 6E and F.

The reapplication of IL-1 β rapidly increased the YFP/CFP ratio again, demonstrating that the reporter is reversible and can be repeatedly activated.

In most experiments in the current study, we used transiently transfected cells that expressed the SAP3K probes at relatively high levels so that we could attain good signal-to-noise ratios. To test if our SAP3K probes also work under more physiologically relevant expression conditions, we generated an HEK293A cell line that stably expressed the YL225 sensor. YL225 is identical to L225 except that it contains YPet instead of Venus. Expression levels of YL225 in individual cells were relatively moderate and were comparable among cells. There were no significant differences in cell shapes or growth rates between YL225-HEK293A and parental HEK293A cells. When YL225-HEK293A cells were stimulated with TNF- α , the YFP/CFP ratio increased rapidly (Fig. 5A and B), indicating that YL225 functioned as an efficient sensor even when it was stably expressed at a moderate level. We next examined if the expression of YL225 might affect SAPK signaling, since the sensor could potentially exert a dominant-inhibitory effect by abortively binding to upstream activators. For that purpose, we compared the stress-induced activations of endogenous SAP2Ks (MKK3/6 and MKK4) and SAPKs (p38 and JNK) in YL225-HEK293A and parental HEK293A cells by immunoblotting using phospho-specific antibodies. When challenged

with sorbitol or anisomycin, both SAP2Ks (MKK3/6 and MKK4) and SAPKs (JNK and p38) were similarly activated in both YL225-HEK293A and HEK293A cells (Fig. 5C), demonstrating that the perturbation of endogenous SAPK signaling by YL225 was negligible.

Temporal dynamics of SAP3K activation in single cells. We then used L225 or YL225 to analyze temporal changes in SAP3K activity in single cells. As measured by immunoblotting, the translation inhibitor anisomycin rapidly increased the level of phosphorylated MKK3 and MKK6, reaching a plateau at 40 min, whereas MMS did so more slowly and reached a maximum at about 2 h (Fig. 6A and B). Immunoblot analysis, however, cannot discern whether SAP3Ks are activated in all cells or in only a subpopulation, nor can it distinguish whether the cells responded in a synchronous or in a stochastic manner. The L225 and YL225 FRET reporters, in contrast, allow these distinctions to be made. When the cells were stimulated by anisomycin, the YFP/CFP ratio in all cells increased synchronously for about 40 min, when it reached a plateau (Fig. 6C and E). The average time taken to reach 50% activation was 20.8 ± 2.7 min. In contrast, when cells were stimulated with MMS, SAP3K activation occurred more slowly and asynchronously (Fig. 6D and F). The average time to reach 50% activation was 97.4 ± 29.5 min. In either case, the YFP/CFP ratio changed in all cells over the observed time period (60 min for

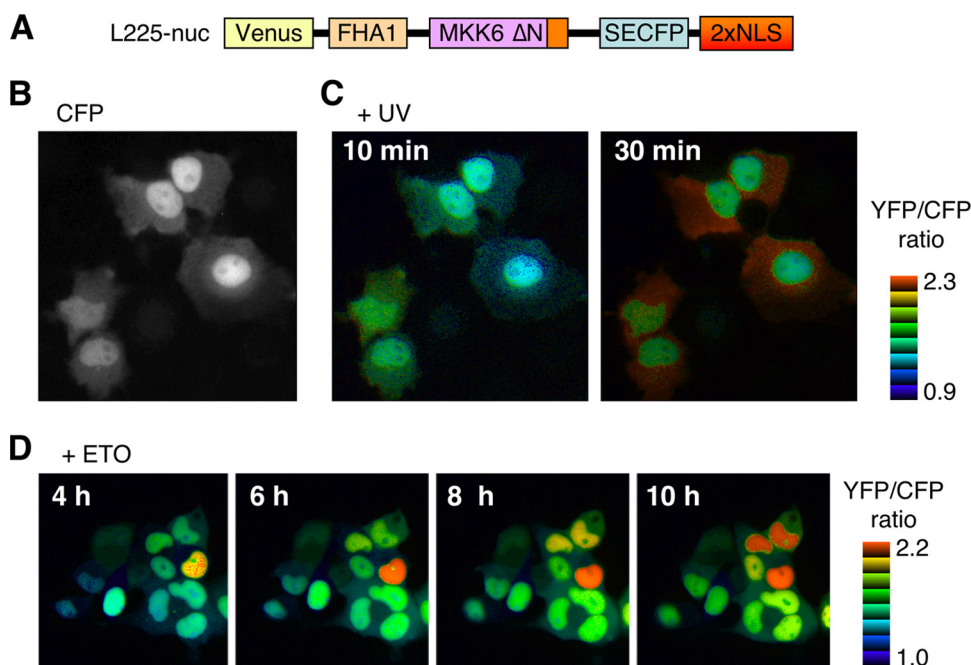


FIG. 8. Etoposide, but not UV-C, activates SAP3Ks in the nucleus. (A) Schematic diagram of the nucleus-directed SAP3K probe L225-nuc, which contains tandem NLS sequences (2xNLS) at the C terminus. (B) Subcellular localization of YL225-nuc. The L225-nuc probe was transfected into COS-7 cells, and the blue-green fluorescence of SECFP was monitored by epifluorescence microscopy. An intense fluorescence of SECFP was observed only in the nuclear region. (C) Cell images of the L225-nuc YFP/CFP emission ratio. Two images from images that were continuously acquired every minute starting at 10 min after UV-C irradiation (80 J/m² UV-C) are shown. (D) COS-7 cells coexpressing L225-nuc and L225-cyt were treated with 100 nM etoposide (ETO) (100 nM; continuous exposure in minimal essential medium with 10% FCS). Cell images of the YFP/CFP emission ratio at the indicated times following the start of etoposide treatment are shown.

anisomycin and 180 min for MMS). The average time course of the YFP/CFP ratio change in both cases (Fig. 6E and F) was comparable to the time course of MKK6 phosphorylation measured by immunoblotting (Fig. 6A and B).

Other stimuli that we examined (UV-C, sorbitol, TNF- α , and EGF) activated SAP3K rapidly and synchronously (Fig. 7A to D). To more easily compare differences among stimuli, the distribution of the times at which the half-maximum FRET increase was reached in individual cells was plotted (Fig. 7E). The slow and stochastic activation of SAP3K by MMS is consistent with an indirect activation mechanism that requires the stress-induced synthesis of an activator protein such as GADD45 (9, 22). In contrast, the fast and synchronous activation of SAP3K by anisomycin and several other stimuli suggests that they activate SAP3K by a more direct mechanism(s).

SAP3K activity reporters directed to specific subcellular loci. The molecular mechanisms by which various stress stimuli activate SAP3Ks are not well understood. To address this problem from a new perspective, we visualized where in the cell various stresses activate SAP3Ks. To improve spatial resolution, we restricted the diffusion of the reporter by targeting it to a specific subcellular area. Specifically, we generated two additional versions of the MAP3K reporter: L225-nuc, which localizes to the nucleus, and L225-pm, which is directed to the plasma membrane. The original L225 reporter, which is cytoplasmic, will be referred to as L225-cyt.

For the generation of L225-nuc, tandem NLSs were attached to the C terminus of the reporter (Fig. 8A). L225-nuc was found mostly in the nucleus, as designed (Fig. 8B). How-

ever, upon UV-C stimulation, we did not detect any activation of L225-nuc in the nucleus (Fig. 8C). The only significant FRET increase was observed in the cytoplasm, where a small amount of L225-nuc was localized. In contrast, when COS-7 cells coexpressing L225-cyt and L225-nuc were treated with the DNA-damaging agent etoposide, the YFP/CFP ratio increase occurred mainly in the nucleus (Fig. 8D). The change in the YFP/CFP ratio induced by etoposide was relatively slow and was asynchronous within a cell population. Thus, the dynamics of activation of SAP3Ks by UV-C and etoposide are different both temporally and spatially, suggesting that their activation mechanisms are fundamentally different.

For the generation of L225-pm, the membrane-targeting CAAX motif of K-ras was attached to the C terminus, and the more FRET-efficient YPet (12) was used in place of Venus. The YPet version of L225-cyt and L225-pm are termed YL225-cyt and YL225-pm, respectively (Fig. 9A). When YL225-cyt and YL225-pm were individually expressed in COS-7 cells, they localized at the expected subcellular loci (data not shown). Furthermore, when they were individually coexpressed with constitutively active MTK1ΔN, their emission spectra changed similarly as the levels of MTK1ΔN expression increased (Fig. 9B).

When both YL225-cyt and YL225-pm were coexpressed in single COS-7 cells and stimulated by cotransfected MTK1ΔN, a strong FRET signal from the entire cell body was observed (Fig. 9C). We then incorporated the nonphosphorylatable T211A mutation (T/A) into one or the other of the reporters. When YL225-cyt-T/A and YL225-pm were coexpressed, the FRET signal was derived mainly from the cell periphery (Fig.

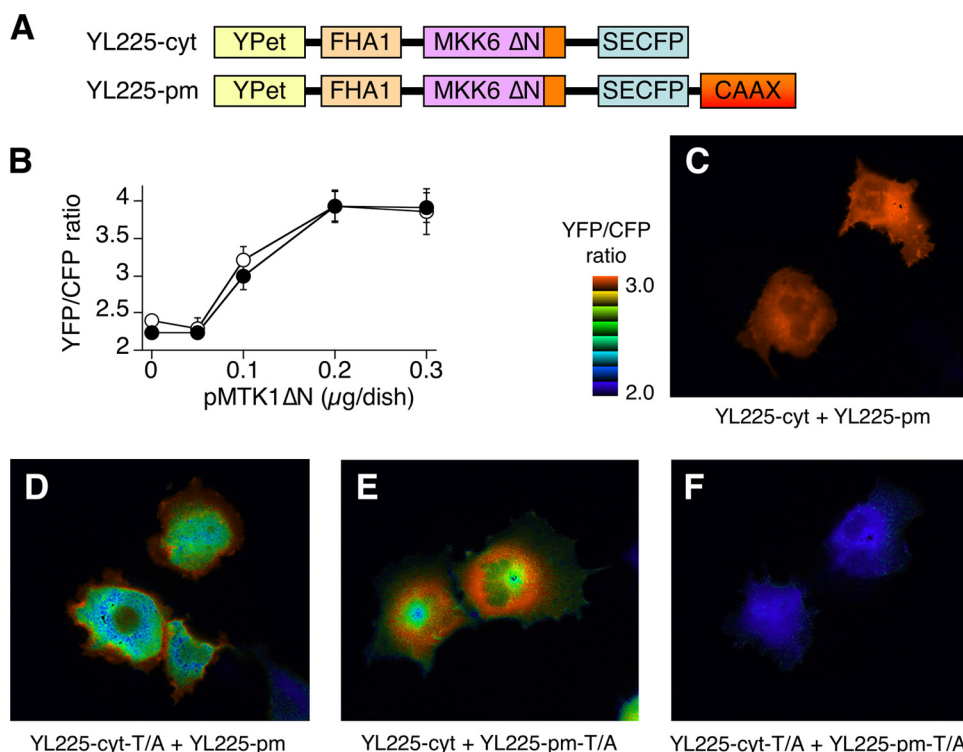


FIG. 9. Epifluorescence FRET imaging discriminates plasma membrane-localized signals from cytoplasmic signals. (A) Schematic diagrams of the cytoplasm-directed SAP3K reporter YL225-cyt and the plasma membrane-directed SAP3K reporter YL225-pm. (B) Dose-dependent changes in the YFP/CFP ratios of YL225-cyt (closed circles) and YL225-pm (open circles). The means $\pm$ SEM (n = approximately 9 to 32) were plotted. (C) COS-7 cells triply coexpressing YL225-cyt, YL225-pm, and the active form of MTK1 (MTK1ΔN) were imaged by epifluorescence microscopy, and the images of the YFP/CFP ratios are shown. (D to F) Either YL225-cyt, YL225-pm, or both were substituted by the corresponding inactive reporter that contains the MKK6-T211A mutation (T/A). A gradient of FRET signals, higher in the peripheral zone (red) and lower in the central area (blue-green), was observed when the cytoplasm-directed reporter was inactive (D). The converse gradient with a centrally higher FRET signal was observed when the plasma membrane-directed reporter was inactive (E). When both reporters were inactive, no signal was detected (F). No significant signal was observed in the nucleus in all cases.

9D). This result indicates that only the plasma membrane-directed reporter (YL225-pm) was activated, because the thin peripheral regions are rich in plasma membrane and poor in cytoplasm, whereas the thick central region is enriched in cytoplasm. Conversely, when YL225-cyt and YL225-pm-T/A were coexpressed, the FRET signal was derived mainly from the central region of the cells (Fig. 9E). When both reporters had the T/A mutation, no FRET signal was observed (Fig. 9F). These data establish the feasibility of distinguishing SAP3K activity in the cytoplasm from that on the plasma membrane.

Spatiotemporal dynamics of SAP3K activation in single cells. When COS-7 cells that coexpressed YL225-cyt and YL225-pm were stimulated by EGF, the FRET signal at the cell periphery increased much faster than did the signal in the central region (Fig. 10A). When HeLa cells that expressed either YL225-cyt or YL225-pm alone were stimulated with EGF, a significant signal was observed only in cells that expressed YL225-pm (Fig. 10B). In contrast, stimulation by TNF- α induced a similar FRET increase in both YL225-cyt-expressing and YL225-pm-expressing cells (Fig. 10C). Thus, EGF preferentially activates the SAP3K pathway on or near the plasma membrane, which is consistent with the membrane localization of the EGF receptor and with the subsequent activation of SAP3K on the membrane. In contrast, TNF- α

activates SAP3Ks both in the cytoplasm and on the membrane, consistent with the two-stage signaling mechanism in which a signaling complex containing MEKK1 (a SAP3K), TRAF2/3, and other proteins is assembled on a membrane-associated TNF receptor, but MEKK1 is activated only after the complex is released from the receptor (7).

As shown in Fig. 11A (left), osmstress (0.4 M sorbitol) preferentially activated YL225-pm. Because the osmstress conditions that we used cause significant changes in cell shape and cell architecture, it was possible that the observed FRET changes were imaging artifacts due, for example, to cell height changes, which were independent of the activating phosphorylation at Thr-211. We therefore determined if the phosphorylation site mutants (T211A) of YL225 and YL225-pm could produce apparent FRET changes by sorbitol. Cells coexpressing YL225-T/A and YL225-pm-T/A showed no FRET increases either in the cytoplasm or on the membrane upon sorbitol stimulation (data not shown), clearly demonstrating that the sorbitol-induced FRET change properly reflects the phosphorylation state of the sensor rather than a stress-induced nonspecific effect. To visualize the spatial time course of SAP3K activation by osmstress, FRET images were taken from a single cell at 1-min intervals. A thin radial section (15 pixels wide) was sliced at each time point, and these sections were stacked in chronological order (Fig. 11B).

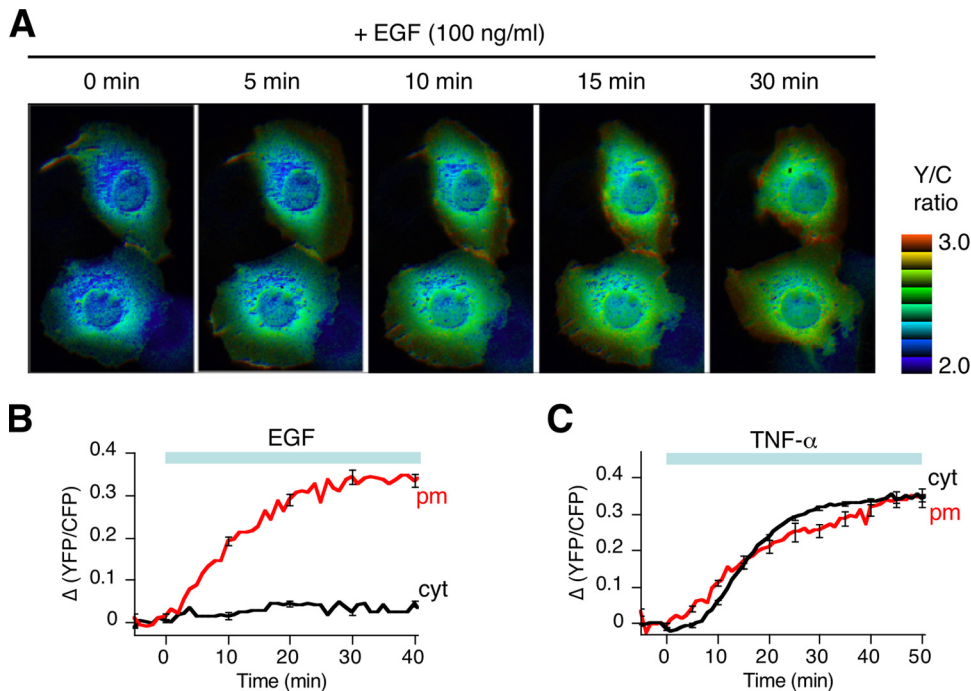


FIG. 10. Activation of SAP3K at the cell peripheries by EGF. (A) COS-7 cells coexpressing both YL225-cyt and YL225-pm were stimulated with EGF. Images of the YFP/CFP ratio (Y/C) were taken immediately before (0 min) and at the indicated times after EGF (100 ng/ml) stimulation. (B and C) HeLa cells expressing either YL225-cyt (cyt) or YL225-pm (pm) were stimulated with either EGF (100 ng/ml) or TNF- α (10 ng/ml). Changes in the YFP/CFP ratio (Δ YFP/CFP) in individual cells were monitored by epifluorescence FRET imaging, and averages for approximately 9 to 19 cells were plotted. Error bars represent SEM.

From this montage, it can be easily seen that SAP3K activation first occurs near the plasma membrane about 10 min after osmo-stress and gradually spreads into the cell interior. Although the mechanism of osmosensing by mammalian cells is only incompletely understood, osmosensing likely takes place on the plasma membrane (23). In clear contrast, when cells were stimulated by the ribotoxin anisomycin, the FRET signal was higher in the central region and weaker at the periphery (Fig. 11A, middle, and 11B). This spatial pattern is consistent with the idea that the translation inhibitor initiates the SAP3K cascade where ribosomes are located, namely, in the cytoplasm.

UV-C preferentially activates SAP3K in the cytoplasm. The molecular mechanism by which UV-C activates SAP3Ks is controversial. Both UV-induced clustering and activation of cell surface receptors (15, 16) and the stalling of the ribosomal translation machinery (5) have been proposed to be mechanisms of the UV-induced activation of SAP3Ks.

We therefore examined the subcellular location at which UV-C irradiation most intensely activates SAP3Ks. The results (Fig. 11A, right, and B) unambiguously indicated that UV-C preferentially activates SAP3Ks in the cytoplasm. These results indicate that UV-C activates the SAPK cascade via cytoplasmic UV sensors, most likely ribosomes, rather than membrane-associated receptors.

DISCUSSION

In this study, we developed a SAP3K activity reporter based on the previously successful protein kinase A, protein kinase C, and Src reporters (24, 26, 27). As in the above-described

probes, the SAP3K-specific phosphorylation of the specific substrate sequence (MKK6 Thr-211) leads to the binding of the phosphorylated threonine to the FHA1 domain in the reporter, which modulates intramolecular YFP-CFP interactions. Significant modifications were necessary, however, to construct an efficient SAP3K reporter, the most critical of which was incorporation into the reporter of the DVD docking site from MKK6 that is necessary for specific binding to SAP3Ks. For many kinases, their substrate specificity is determined not only by the substrate sequence around the phosphorylation site but also by a specific interaction with a docking site. We have previously shown that the DVD docking site (24 aa) derived from the C terminus of MKK6 binds strongly and specifically to SAP3Ks (22). More importantly, in the absence of this DVD docking site, or in the presence of a mutant DVD docking site, MKK6 cannot interact with any SAP3K that would otherwise phosphorylate and activate MKK6 (8, 22). Thus, the docking interaction is absolutely essential for activated SAP3Ks to phosphorylate MKK6. Indeed, a DVD docking-site mutation completely abolished the response of L225 to activating SAP3Ks. Thus, our data show that the proper inclusion of a docking site is a key element for the successful development of specific kinase reporters.

An important feature of a kinase reporter is its specificity. The L225 SAP3K reporter, which has both the phosphorylation site and the DVD docking site of MKK6, can be used to monitor the combined activities of all of the kinases that activate MKK6, including MTK1, MEKK1, TAK1, ASK1, and TAO2, but does not respond to Raf family MAP3Ks. Thus, the

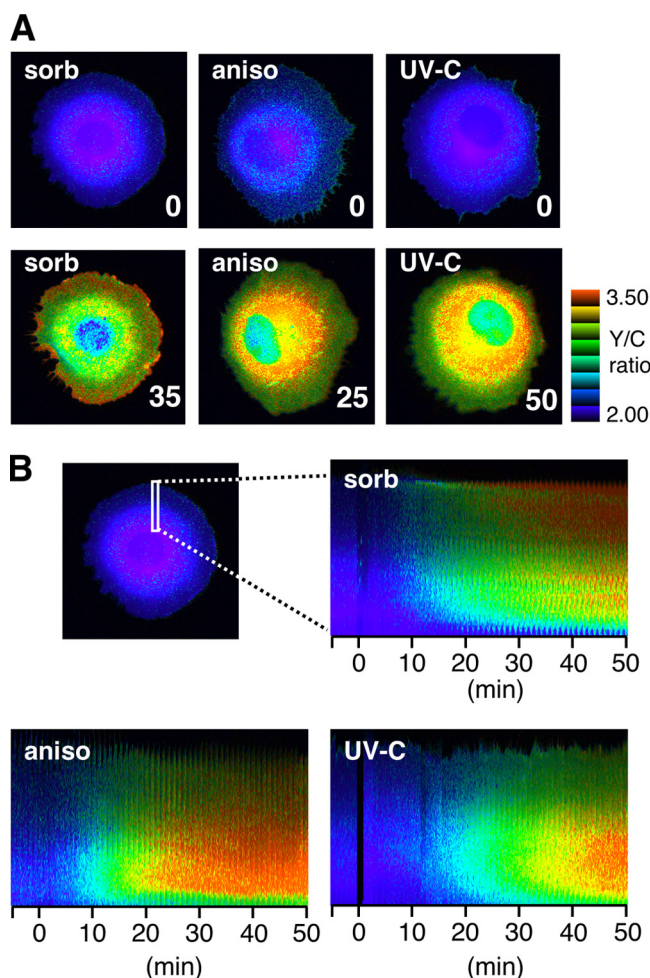


FIG. 11. Spatiotemporal dynamics of SAP3K activation in single cells. (A) Subcellular distributions of SAP3K activities in COS-7 cells challenged by hyperosmotic stress (0.4 M sorbitol [sorb], continuous exposure), ribotoxic stress (10 ng/ml anisomycin [aniso], continuous exposure), and UV irradiation (80 J/m² UV-C, irradiated at time zero) were imaged. Numbers in the panels are times (minutes) after stimulation. (B) Propagation of stress-induced SAP3K activities as visualized by montages of images of YFP/CFP (Y/C) ratios of the white rectangular region along the radius from the nucleus (bottom) to the cell periphery (top). Cells were stimulated at 0 min, and images were continuously obtained every minute.

L225 reporter is specific to the group of stress-activated SAP3Ks that interact with MKK6.

The activation time course of the L225 SAP3K reporter is consistent with the phosphorylation time course of endogenous MKK6. Thus, L225 can be used to closely monitor the activation state of endogenous SAP3Ks. However, more caution will be necessary to interpret the deactivation kinetics. Although the L225 reporter is reversible, as shown by the reversal of the FRET signal when the stimulus was eliminated (Fig. 4), L225 might not be dephosphorylated as efficiently as endogenous MKK6, because phosphorylated Thr-218 in L225 is protected by the bound FHA1 domain. Employing a lower-affinity mutant of the FHA1 domain might improve the deactivation kinetics, but it will also reduce the FRET signal for activation.

Using the SAP3K reporter, we revealed two previously un-

known properties of SAP3K activation that are of major importance for an understanding of SAPK signaling pathways. First, we found that the activation time course of SAP3Ks within a cell population is either synchronous or heterogeneous depending on the type of stress. When cells were stressed by anisomycin, all cells in a population synchronously activated SAP3Ks, and SAP3K activation occurred relatively rapidly, reaching half-maximum activation at about 20 min. Anisomycin inhibits the peptidyl transferase activity of the ribosome and thus inhibits protein synthesis in the elongation phase (14). Although the mechanism by which a blocked ribosome activates a SAP3K is unknown, it clearly cannot involve the neosynthesis of a protein such as a SAP3K activator. Therefore, it is more likely that a blocked ribosome itself, or a released ribosomal component, directly activates a SAP3K or an upstream activator of a SAP3K. The synchronous and rapid time course of SAP3K activation revealed by our reporter is consistent with this idea. UV-C, osmotic stress, TNF- α , and EGF also activated SAP3Ks rapidly and synchronously. We further found that EGF activated SAP3Ks less vigorously but slightly faster than the other stresses that we tested (Fig. 7D and E). The reason for this difference is not clear at present.

In contrast to the effect of these stresses, MMS activated SAP3Ks much more slowly, requiring 100 min to achieve average half-maximum activation. Furthermore, activation time courses within the population of MMS-stressed cells were quite heterogeneous. The slower time course suggests that MMS activates SAP3K by a more indirect mechanism than other stresses. One possibility is that MMS may rely on the accumulation of an activator protein like GADD45 for the activation of SAP3K (9, 21). Alternatively, the different activation time courses within the cell population might be related to the cell cycle. For example, it is possible that cells in a certain cell cycle stage are more susceptible to MMS activation than cells in other cell cycle stages.

The second novel finding of this study was that different stresses initiate SAPK signaling at different cellular loci. Using a combination of a plasma membrane-associated reporter and a cytoplasmic reporter, we could clearly distinguish whether the initial activation of SAP3Ks occurs on the plasma membrane or in the cytoplasm. SAP3K activation by EGF, osmotic stress, and anisomycin took place at the expected location. Thus, EGF activated SAP3Ks on the plasma membrane where EGF receptors are located, consistent with the fact that growth factors weakly activate the SAPK cascades. Even in the absence of any growth factor stimulation, weak SAP3K activation was observed in the area of cell-cell contact, possibly via integrin-mediated signaling (0 min) (Fig. 10A). Osmotic stress also activated SAP3Ks on the plasma membrane where osmosensors are presumably located (23). In contrast, anisomycin activated SAP3Ks in the cytoplasm. This corroborates the view that blocked ribosomes in the cytoplasm initiate the activation of SAP3Ks.

The L225 reporter was also useful for revealing where and how SAP3Ks are activated by other stresses such as UV-C. Thus, the L225 reporter clearly demonstrated that UV-C activated SAP3Ks in the cytoplasm, thereby unambiguously supporting the model that UV activates the SAPK pathway in the cytoplasm by damaging ribosomes rather than by the clustering of cell surface receptors. The precise mechanism by which the

damaged ribosomes activate SAP3Ks remains to be determined.

In summary, we described a specific and sensitive reporter for SAP3K activation that can be used for real-time analysis of SAP3K activation in live single cells. The reporter can distinguish the cellular locale of SAP3K activation following different stresses. This reporter has wider applicability than the ones reported here. For example, it should also be an excellent tool for high-throughput screening of modulators of SAPK signaling, which are of potential clinical importance for the development of anti-inflammatory and antitumor drugs. Furthermore, it will be possible to use this reporter, with some modifications, to monitor SAP3K activation in live organisms or in developing embryos.

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