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Control of Antigen Presentation with a Photoreleasable Agonist Peptide

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T cells survey target cells by scanning for antigenic peptides presented by major histocompatibility complex molecules (pMHC) with T cell receptor (TCR).[1] Engagement of pMHC by TCR induces a cascade of signaling events, including a rise in intracellular calcium levels and the formation of a large scale spatial pattern of signaling and adhesion molecules called the immunological synapse (IS).[3] Recent studies suggest a direct role for the spatial pattern of molecules in the synapse with respect to regulation of TCR signaling.[4−6] The interaction between TCR and pMHC is sensitive to point mutations in the antigenic peptide; mutated agonist peptides induce a panoply of responses in the T cell ranging from attenuated activation to antagonism to null.[7] In the well studied TCR and pMHC pair of AND and MCC−IE^k (moth cytochrome *c*, residues 88−103),[8,9] replacing the critical lysine at position 99 with glutamate (and thus flipping the charge) diminishes the agonist effect of the peptide. On the basis of the crystal structure of the MCC−IE^k and its putative interaction with AND TCR,[2] we hypothesized that the presence of a bulky caging group on the end of the critical K99 residue of MCC would abolish activation (Figure 1). Caged proteins and peptides are powerful tools for controlling cellular signaling. 6-Nitroveratryloxycarbonyl (NVOC) was originally introduced as a protecting group for solid-phase peptide synthesis[10] and has since been employed in a variety of spatially and temporally modulated chemical reactions, in particular involving amino acids and peptides.[11,12] It can be readily linked to terminal amines and is cleaved by near-UV light. Here we show that caged antigenic peptide can be used to selectively activate T cells in a spatially and temporally controlled fashion. This strategy provides a straightforward way to pattern two-dimensional arrangements of molecules for probing the spatial regulation of T-cell signaling.

A peptide variant of MCC in which the Lys99 residue critical to MHC−TCR interaction was derivatized with NVOC was synthesized using solid-phase peptide synthesis (Figure S1).[13] The UV-light-induced uncaging rate of the peptide was determined by mass spectrometry (Supporting Information, Figure S2). All experiments were performed in the presence of 50 μM dithiothreitol (DTT) to decrease secondary reactions of the nitrosoacetophenone photoproduct which can chemically cross-link proteins.[14] The addition of 50 μM DTT did not interfere with protein stability. The basal level of uncaged MCC contamination due to sample manipulation in dimmed room light was determined to be ∼1%. Uncaging was also confirmed with the TCR-mimic antibody D4 (Figure S3).

The functionality of NVOC−MCC with live T cells was examined. T-cell blasts were plated on planar bilayers containing mobile intercellular adhesion molecule-1 (ICAM) and peptide-loaded IE^k. Planar bilayers are well established in the study of the immunological synapse and are stable over the course of days.[3]

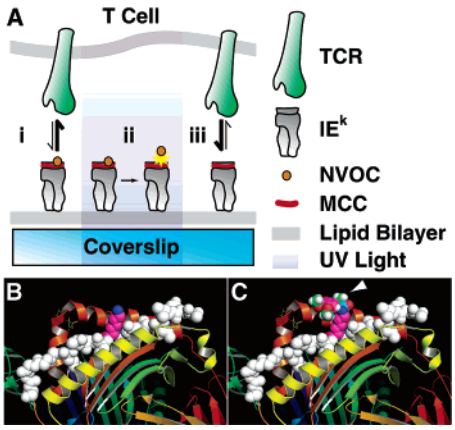

Figure 1. Experimental setup: (Ai) T cell receptor (TCR) weakly binds moth cytochrome *c* (88−103) (MCC) peptide presented by IE^k major histocompatibility complex (IE^k) when the peptide is derivatized on Lys99 with the light sensitive protecting group 6-nitroveratryloxycarbonyl (NVOC). (Aii) Near-UV light cleaves the protecting group, restoring (Aiii) strong binding between TCR and MCC−IE^k. (B) The crystal structure of MCC−IE^k,[2] shown in ribbon form, underscores the importance of Lys99, highlighted against the white space-filling MCC peptide. (C) A cartoon of NVOC derivatized MCC peptide illustrates NVOC mediated steric hindrance of TCR binding to MCC−IE^k.

Because of the uncaged peptide contamination, it was necessary to dilute the MCC or NVOC−MCC in a background of the non-activating peptide MCC−T102E, in which the threonine at position 102 has been replaced by glutamate.[3] The surface density ratio of activating to caged MCC−IE^k could not be determined directly; assuming that caging K99 does not modify the binding constant of MCC to IE^k, a 1:25 dilution of MCC/MCC−T102E with a surface density of 50 IE^k/μm^2 and a loading efficiency of ∼50% yields an approximate surface density of 1−2 caged peptides/μm^2, which is above the activating threshold for MCC on bilayers.[3] The amount of uncaged MCC impurity in NVOC−MCC experiments was determined to be approximately 0.02/μm^2, which is subthreshold (B. Rossenova, personal communication). At this very low density of NVOC−MCC, at most 200 molecules of the photoproduct nitrosoacetophenone are generated in the vicinity of a cell during uncaging. Any molecules not immediately quenched by the DTT in solution would nonspecifically interact with cell surface molecules, and thus it is unlikely that the photoproducts produce any cellular effect.

Intracellular calcium flux, which occurs downstream of TCR binding in T-cell activation, was examined in cells stimulated with agonist, caged and self-peptide. T-cell blasts were loaded with the calcium-sensitive dye Fluo-Lojo and then plated onto peptide-displaying bilayers. Cells adopted a rounded morphology and fluxed calcium when stimulated with MCC bilayers (Figure 2). On NVOC−MCC bilayers, cells crawled, showing a low level of intracellular calcium similar to cells on T102E bilayers. NVOC−

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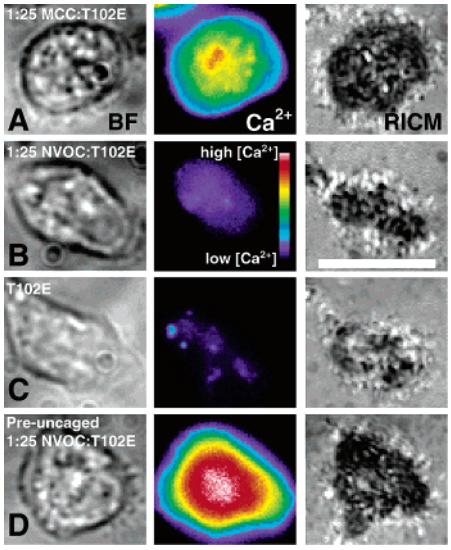

Figure 2. UV light restores agonist activity of caged peptide. (A) Cells loaded with the calcium-sensitive dye Fluo-Lojo strongly flux calcium when stimulated by bilayers displaying the agonist peptide MCC diluted in a background of the self-peptide T102E. (B) When plated on bilayers displaying caged NVOC−MCC peptide in the same dilution, cells do not flux calcium and adopt a crawling morphology, similar to the response (C) of cells to bilayers displaying only the self-peptide T102E. (D) Pre-irradiation of NVOC−MCC loaded bilayers with UV light (15 min, 0.5 nW/μm^2, 97% uncaged) rescues the stimulatory effect of the caged peptide. Cell adhesion was confirmed with reflection interference contrast microscopy; dark regions indicate close adherence to the bilayer. Scalebar is 10 μm.

MCC bilayers exposed to UV light before the addition of cells behaved similarly to MCC bilayers. Extended illumination with >450 nm light did not uncage the NVOC, indicating visible light fluorescence imaging is compatible with the caged peptide. These data suggest that UV light successfully exposes the original MCC peptide from NVOC−MCC in situ.

Caged peptide also affects immune synapse formation. Cells that were stimulated with bilayers loaded with 1:25 MCC/T102E strongly tended to stop and form the characteristic immune synapse pattern of a strong central accumulation of TCR surrounded by a ring of ICAM (Figure 3A). In contrast, cells stimulated with bilayers displaying NVOC−MCC did not form synapses. TCR was diffuse over the face of these cells, whereas activated cells displayed the classic central accumulation of TCR. The aggregate behavior is similar to that of cells stimulated with empty IE^k or MCC−T102E loaded IE^k, indicating that cells were not activated by caged peptide.

Exposure to UV light was sufficient to activate T cells on bilayers displaying caged MCC−IE^k. These results are shown for a typical cell in Figure 3B. A migrating cell was irradiated with UV light at ~80 nW/μm^2 for 60 s, sufficient to release essentially 100% of the caged peptide. Before uncaging, the cell displayed the hand-mirror morphology and wedge-shaped ICAM distribution of a migrating cell. After uncaging, the cell stopped, rounded up, and formed the ring of ICAM characteristic of the immune synapse, indicating activation of the TCR signaling cascade. A typical cell on a control bilayer displaying only the null peptide MCC−T102E is shown in Figure 3C. After irradiation, the cell continues to crawl, unaffected by the UV exposure. Moreover, the UV light does not appreciably bleach the Cy5 labeled ICAM, indicating that the caging group is compatible with standard fluorescence imaging. UV light directed onto a T cell already crawling on the surface sometimes did not induce activation. These T cells were probably in an internal state unreceptive to activation, possibly owing to mild antagonism by MCC−T102E.

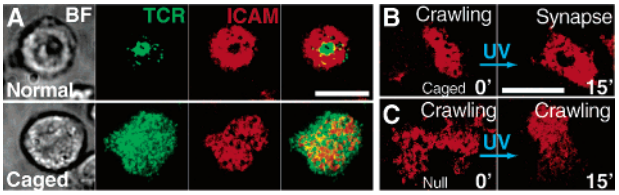

Figure 3. Control of T-cell signaling with caged peptide. (A) Cells plated on bilayers displaying IE^k and ICAM-1 loaded with normal MCC form the classic immunological synapse, with a central accumulation of TCR surrounded by a peripheral ring of ICAM-1. Cells stimulated with caged NVOC−MCC peptide display diffuse TCR and ICAM. (B) A migrating cell displaying the stereotypical hand-mirror shape and ICAM wedge was irradiated with UV (80 nW/μm^2, 60 s). Upon UV exposure, the cell rounded up and formed the classic ICAM ring, indicative of synaptic signaling (signal onset was within 5 min). (C) On bilayers displaying only the null peptide MCC−T102E, UV irradiation does not cause any signaling. At 0 min, a crawling cell was irradiated with UV (80 nW/μm^2, 30 s). Just after irradiation, the cell retained the hand-mirror morphology. At 15 min, the cell continues to crawl on the bilayer, indicating no damage or signaling. Scalebars are 10 μm.

NVOC−MCC is an effective reagent for specific activation of T-cell signaling on supported bilayers. Using a standard epifluorescence microscope, T-cell signaling and immune synapse formation can be activated in a spatially and temporally controlled fashion. Future applications of photoreleasable peptide are most promising in the area of subcellular protein patterning. By combining nanofabricated barriers to diffusion with photolithography of activating peptide, study of the effects of subcellular juxtaposition of agonist and nonactivating peptide signals on T-cell activation will be directly accessible.

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Supporting Information Available: Experimental procedures and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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