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Combining Surface Chemistry with a FRET-Based Biosensor to Study the Dynamics of RhoA GTPase Activation in Cells on Patterned Substrates

Louis Hodgson,[†] Eugene W. L. Chan,[§] Klaus M. Hahn,[†] and Muhammad N. Yousaf*[,§]

Department of Chemistry, Carolina Center for Genome Science, and Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3290

Received May 2, 2007; E-mail: mnyousaf@email.unc.edu

RhoA is a member of the Rho family, a subset of the Ras superfamily of GTP-binding proteins.[1] RhoA is of central importance in the regulation of contractility[3] and cell interaction with the extracellular matrix.[4] It serves as a molecular switch that cycles between a GDP-bound inactive state and a GTP-bound active state.[2] We recently developed a FRET-based biosensor that revealed precise timing and localization of RhoA activation relative to cell protrusion and adhesion.[5] In particular, we observed elevated levels of RhoA activity concentrated in a sharp band at the edge of protrusions as cells migrated randomly on fibronectin-coated substrates. This and other studies suggested that extracellular cues from surface-bound ligands modulate RhoA signaling in membrane protrusions. To investigate further the spatio-temporal dynamics of RhoA activation as regulated by extracellular matrix interactions, we set out to use model substrates with more sophisticated and flexible surface properties.[6] These studies would greatly benefit from two surface parameters: (1) the presentation of extracellular ligands at precisely defined locations relative to cell extensions; (2) the model substrate must be compatible with high-resolution *live-cell* fluorescence microscopy. The microscopy has presented a major technical obstacle, as biosensors in living cells must be used at low intracellular concentrations that produce relatively low light levels; gold model substrates are known to be highly absorptive at the wavelengths used for *live-cell* fluorescence imaging, causing efficient quenching of the fluorescent molecules, significantly reducing fluorescence excitation. In this communication, we combine a surface chemistry approach with our FRET-based biosensor of RhoA activation to investigate effects of cell microenvironment on RhoA activation in membrane protrusions. The approach is based on the combination of self-assembled monolayers (SAMs) of alkanethiolates on gold to generate defined hydrophobic and hydrophilic regions on the model surface and sensitive live-cell fluorescence microscopy that is compatible with gold surfaces.

We first used microcontact printing to pattern hexadecanethiol on gold-coated glass substrates (Figure 1). The remaining bare gold regions were backfilled with tetra(ethylene glycol) alkanethiol to prevent nonspecific cell adhesion.[7] Immobilization of fibronectin to the hydrophobic pattern promotes ligand-mediated cell adhesion on a selected region of the monolayer substrate.[8] The adherent cells remain confined within the patterns by the inert tetra(ethylene glycol) monolayers but can extend filopodia and lamellapodial protrusions to sample the external environment. Because there is a lack of surface-bound adhesive ligands outside the cell patterns, the cells are unable to establish traction on the nonadhesive inert region of the monolayer, resulting in membrane retraction. The complex activation and inactivation of RhoA at the leading edge regulate adhesion, actin assembly and disassembly, and actin contractility. It is critical for generating cell polarity and cell migration. To visualize the location and kinetics of RhoA activation, we used a genetically encoded FRET biosensor stably expressed in mouse fibroblasts.[5] We examined how changes in the microenvironment, from regions that support or do not support adhesion, influence RhoA activation in protrusions. Until now, access to the powerful live-cell fluorescence microscopy techniques on SAM gold surfaces was difficult due to the efficient quenching absorbance of the low-intensity fluorescence within cells' excitation wavelengths by the underlying gold surface. To overcome this severe limitation, we developed a unique inverted live-cell chamber (schematic in Supporting Information) for live-cell high-resolution microscopy. This is the first study combining live-cell FRET and SAMs on gold surfaces.

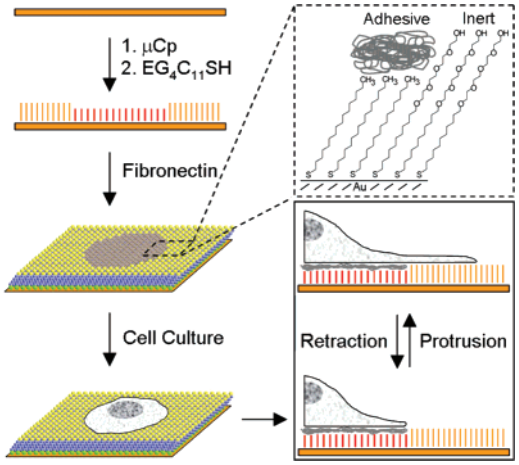

Figure 1. Examining RhoA activation using the FRET biosensor in cells on patterned self-assembled monolayers. Hexadecanethiol was patterned into 130 μm circles on gold-coated glass substrates by microcontact printing. The remaining bare gold was backfilled by tetra(ethylene glycol) alkanethiol to provide the inert region. The monolayer substrate was then coated with fibronectin to promote cell adhesion on the hydrophobic patterns. Addition of fibroblasts to the patterned SAMs resulted in cell attachment only to the hydrophobic region of the surface. The cells showed dynamic protrusion and retraction of lamellapodia from the adhesive region over the inert region of the surface. The cells extended lamellae but were unable to attach and move from the fibronectin-coated region.

Figure 2A shows a schematic representation of the biosensor, which consists of a Rho-binding domain (RBD)[9] that binds selectively to the activated, GTP-bound form of RhoA. Upon activation by GTP loading, the binding of RBD to Rho modifies the relative orientation of the two fluorophores and leads to an increase in the FRET signal (Figure 2B). Figure 2C shows a phase contrast micrograph of mouse embryonic fibroblasts (MEF) confined within a fibronectin-coated circular pattern, with the cell extensions sampling the inert region of the monolayer and therefore

[§] Department of Chemistry and Carolina Center for Genome Science.
[†] Department of Pharmacology.

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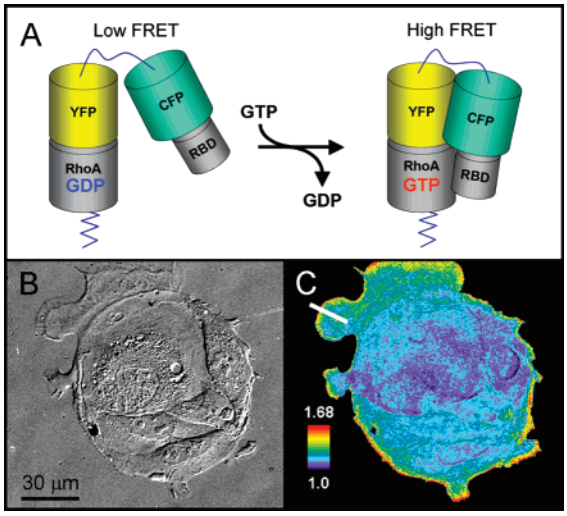

Figure 2. Genetically encoded single-chain biosensor of RhoA nucleotide state. (A) RhoA is fused to citrine YFP followed by ECFP and a small binding domain derived from the RhoA effector protein Rhotekin. Activated RhoA (GTP-bound state) binds the small binding domain and changes the relative orientation between citrine YFP and ECFP, thereby affecting FRET. By monitoring the ratio of FRET and CFP emissions upon CFP excitation, changes in RhoA activation states can be monitored in real time. (B) Differential interference contrast image of mouse embryonic fibroblasts (MEF) stably expressing the RhoA biosensor, confined to a circular pattern of 130 μm diameter. (C) Ratiometric image of FRET emission over CFP emission, with pseudocolor indicating the extent of RhoA activation. The white line drawn perpendicular to the leading edge in (C) is representative of the line scan measurements shown in Figure 3.

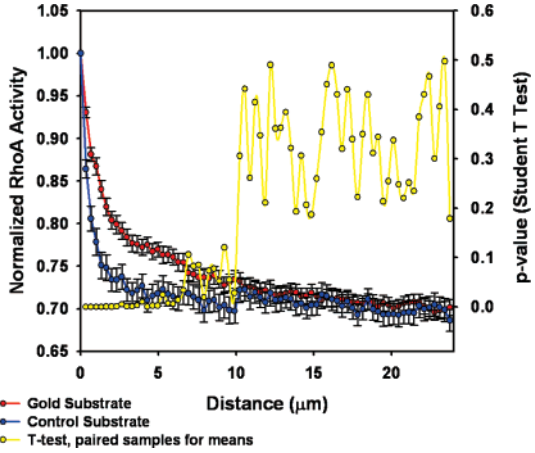

Figure 3. RhoA activity in cell protrusions on gold model substrates, comparing extension over inert monolayer versus fibronectin. RhoA activity is elevated in the absence of lamellae substrate adhesion. Line scans of RhoA activation were taken (two pixel line widths) at the leading edge of protrusions as shown in Figure 2C. Measurements were taken within protrusions that extended past the region of geometric confinement, or over glass coverslips uniformly coated with fibronectin (control substrate). For extensions over inert monolayer, a total of 49 line scans from 5 cells were analyzed at different times and different locations along the leading edge. For the control cells, 49 line scans from a total of 15 cells were analyzed. Raw intensity values were normalized to the leading edge intensity value. Error bars indicate mean ± SEM at each time point. P values shown in yellow indicate significant difference ($P < 0.01$) within a 6 μm distance from the leading edge.

partially extending beyond the adhesive pattern region (see movie in Supporting Information).[10] Figure 2 shows the ratiometric image of FRET emission divided by CFP emission for the cells shown in 2C. We observed that RhoA activity is much higher at the periphery than within the cell body, as is consistent with RhoA activation in cells plated on fibronectin-coated glass substrates.[5]

In contrast to cells plated on fibronectin, the RhoA activation at the cell edge was elevated and did not decrease as it does when the cell establishes new stable boundaries.[5] Figure 3 shows the normalized RhoA activity in protrusions extending over inert region of the SAMs surface (red line) versus protrusions on fibronectin-coated glass substrates (blue line). RhoA activity was elevated over the first 6 μm extending backward from the cell edge. These results suggest a new role for Rho in early phases of extension, which is then modulated by adhesion-induced Rac activation and consequent inhibition of RhoA.

In summary, we have combined a surface chemistry approach with a FRET-based biosensor to demonstrate for the first time how changes in the extracellular microenvironment can affect the dynamics of RhoA activations. By utilizing microcontact printing to spatially define cell adhesive and inert regions on model surfaces, we show a dramatic increase in RhoA activity in cell protrusions over inert regions. We are currently extending this study with dynamic substrates that can alter the surface microenvironment in situ via an applied potential in order to further examine RhoA activation during cell polarity and cell migration.[11]

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Supporting Information Available: Detail schematic drawings of the microscope and live-cell chamber. Phase contrast and FRET movie of MEF cells on patterned SAM gold surfaces. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) The patterned substrates were immersed in an ethanolic solution containing the tetra(ethylene glycol) alkanethiol (1 mM) for 12 h.
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(9) Rho binding domain is a Rho effect protein Rhotekin that interacts only to the GTP-bound Rho. See: Ren, X. D.; Kiosses, W. B.; Schwartz, M. A. *EMBO J.* **1999**, *18*, 578−585.
(10) The mouse fibroblasts are engineered to enable expression of the biosensor at levels similar to endogenous RhoA. The optimal expression level ensures that RhoGDI binding capacity is not overwhelmed, and cell morphology and motile behavior are not altered.
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