J|A|C|S
COMMUNICATIONS

Published on Web 02/20/2002

A New Strategy for Caging Proteins Regulated by Kinases

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Received November 21, 2001

Intracellular signal tranduction pathways serve as portals that transfer information between discrete cellular sites. The primary participants of these pathways, protein kinases, act as information conduits by catalyzing the phosphorylation of serine, threonine, or tyrosine residues in their assigned protein substrates. The phosphorylation event typically generates a dramatic change in the catalytic properties of the substrate. The extraordinary biochemical, spatial, and temporal complexity of signaling pathways has created a demand for sophisticated molecular tools that can tease out the role of individual signaling participants. For example, issues related to both when and where specific enzymes are activated in response to a stimulus and the consequences of their activation can be addressed with photoactivatable ("caged")[1] analogues of these enzymes. Indeed, examples of caged protein kinases[2] and protein phosphatases[3] have recently been described. In these instances, active-site residues have been covalently modified with a photolabile linkage. The resulting inactive enzyme can be then "switched on" by exposure to an appropriate high-intensity light source. However, as noted above, in many instances, enzymatic activity is not so much controlled by the active site as it is by the phosphorylation status at a nonactive site position. In this communication we describe the construction of a protein whose activity is controlled via photochemical uncaging at an ancillary phosphorylation site.

An early molecular event that precedes cell motility is the cofilin-driven generation of actin barbed ends from existing filamentous-actin (F-actin).[4] Multiple cofilin monomers bind cooperatively to F-actin, which induces a twist about the filamentous actin, ultimately severing the polymer chain (Figure 1).[5] The actin barbed ends thus formed can then elongate to form new filaments immediately adjacent to the tip of the leading edge of the cell. As a result, the membrane moves outward and forms a protrusion. Alternatively, the severed F-actin filaments can further depolymerize to individual actin monomers (Figure 1). Cofilin activity is regulated by LIM-kinase, which catalyzes the phosphorylation of cofilin at Ser-3, producing inactive cofilin **2** from its nonphosphorylated active counterpart **1**.[6] Indeed, cofilin mutants that possess a negatively charged Asp or Glu in place of Ser-3 exhibit a dramatic reduction in depolymerization activity compared to native cofilin.[7] In addition, expression of the constituitively active catalytic subunit of the LIM-kinase in MTLn3 cells (metastatic mammary adenocarcinoma) results in the near total phosphorylation of cofilin, which is sufficient to inhibit the appearance of barbed ends and lamellipod protrusion.[8] We constructed a light-activated version of this F-actin severing enzyme to further evaluate the role of cofilin in cell motility.

Caged cofilin was prepared in two stages. First, we constructed a permanently active cofilin by generating a mutant that cannot be phosphorylated at Ser-3 by the LIM-kinase. We, and others, have found that protein kinases will not catalyze the phosphorylation of substrates containing a cysteine residue in place of the phosphorylatable serine moiety.[9] Consequently, a Ser-to-Cys mutant

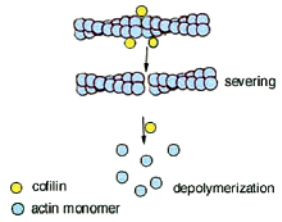
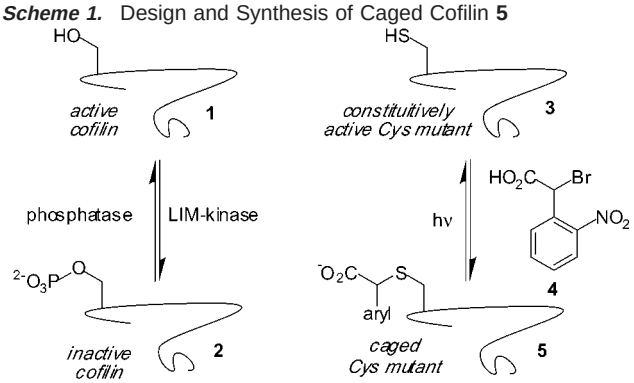


Figure 1. Mechanism of severing and depolymerization of filamentous actin by cofilin.

Scheme 1. Design and Synthesis of Caged Cofilin **5**



cofilin ("Cys-3 cofilin", **3**) was prepared via site-directed mutagenesis. As a control, the nonphosphorylatable Ser-to-Ala mutant ("Ala-3 cofilin") was generated as well. As expected, neither the Cys-3 nor the Ala-3 cofilin mutants serve as LIM-kinase substrates (see Supporting Information). **3** was subsequently chemically modified at Cys-3 with α-bromo-(2-nitrophenyl)acetic acid (**4**).[10] *The latter reaction furnished **5**, a species that electrostatically resembles the inactive phosphorylated wild-type enzyme **2*** (Scheme 1). Although native cofilin contains four Cys moieties, we found that the corresponding Ala-3 cofilin mutant does not react with **4**, suggesting that the four native Cys residues are engaged in disulfide bonds.

The activities of the various cofilin derivatives were assessed by two different assays. The ability of cofilin to sever and subsequently depolymerize actin filaments can be spectrofluorimetrically followed using pyrene-labeled F-actin (Figure 2).[11] A reduction in fluorescence is directly correlated with the production of actin monomers. Cys-3 cofilin displays active F-actin severing activity at a rate that is approximately 70% of that displayed by the constituitively active Ala-3 cofilin mutant (data not shown). By comparison, F-actin in buffer alone slowly depolymerizes as a function of time. In the presence of the chemically modified, caged cofilin **5**, the rate of depolymerization of actin is similar to the rate in buffer alone, indicating that the caged cofilin is not able to depolymerize F-actin. By contrast, approximately 80% of Cys-3

10.1021/ja017592l CCC: $22.00

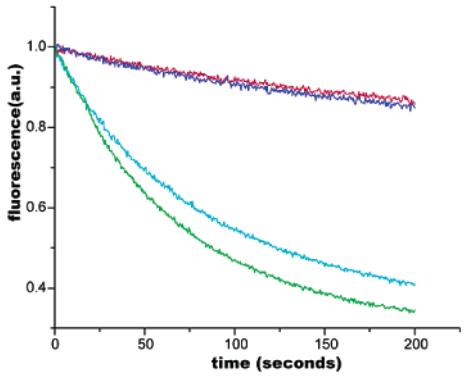


Figure 2. Depolymerization of F-actin by (a) buffer only (red), (b) Cys-3 cofilin **3** (green), (c) caged Cys-3 cofilin **5** (dark blue), and irradiated (30 min) Cys-3 cofilin (cyan). Cys-3 incubated with LIM-K shows the same rate of depolymerization as Cys-3 alone (data not shown). See Supporting Information for experimental details.

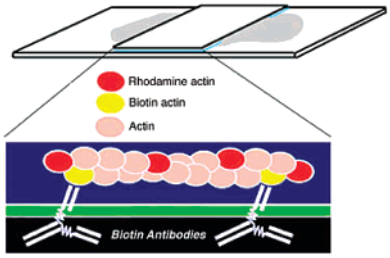


Figure 3. Slide chamber used for severing assay.

cofilin activity is restored upon irradiation[12] of the caged-cofilin **5** (180 W Hg-arc lamp through a 285 nm cutoff filter).

The severing activity of Cys-3 cofilin was further characterized via a light microscope assay under conditions in which only cofilin-induced actin severing is significant (i.e., little or no depolymerization). The latter allowed us to directly assess the severing activity of cofilin without complications from other activities, such as actin monomer/cofilin complex formation or cofilin-enhanced off rates of actin monomers from F-actin. Direct visualization of F-actin was achieved by copolymerizing rhodamine−actin along with native and biotin-labeled actin (Figure 3). These actin filaments were then tethered to a nitrocellulose-coated microscope slide using an antibiotin antibody (Figure 4a). Perfusion of caged cofilin into the slide chamber and subsequent incubation failed to induce any appreciable severing of the fluorescently labeled filaments (see Supporting Information). By contrast, photoirradiation[12] of caged cofilin prior to perfusion and incubation generated multiple cleavage sites along the length of the actin filaments (Figure 4b). Perhaps most reassuring is the fact that as little as 100 nM of light-exposed caged cofilin generates significant F-actin severing activity. This concentration is more than 50 times less than the intracellular concentration of cofilin.[7,11] suggesting that even small jumps in active cofilin levels could have a dramatic influence on cell behavior.

In summary, we have developed a strategy for the design and construction of caged protein participants of signaling pathways.

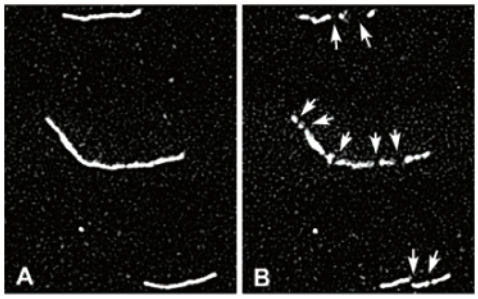


Figure 4. (A) Rhodamine-labeled F-actin filaments (B) in the presence of irradiated (15 min)[12] caged Cys-3 cofilin **5**. Cleavage sites along the F-actin filaments are marked with arrows.

Replacement of the phosphorylatable serine with a nonphosphorylatable cysteine ensures that the protein, once uncaged, cannot be switched off by intracellular protein kinases. Furthermore, incorporation of a negatively charged caging moiety, at the ancillary phosphorylation site, electrostatically mimics the natural serine phosphate present in the inactive wild-type cofilin. Photoremoval of the cage generates a constituitively active enzyme. Work is now underway to examine the spatial and temporal influence of cofilin on cellular dynamics in response to chemotactic agents.

Acknowledgment. We thank the National Institutes of Health for financial support.

Supporting Information Available: Experimental details of the isolation, caging, irradiation, and characterization of Cys-3 cofilin, as well as the depolymerization and severing assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The 15 min irradiation time required for the in vitro photoactivation of caged cofilin should be reduced to <10 s inside a cell. For example, a caged fluorescein dextran is uncaged >120 times faster in vivo than in vitro (data not shown). This is due to a dramatically enhanced laser-driven photon flux through a small cell volume (∼2 pL) see Mitchison, T. J. *J. Cell Biol.* **1989**, *109*, 637−652.

JA017592L