V. 3.2

Rho/Rac/Cdc42 Activator I
Deamidation of Rho Gln-63 & Rac/Cdc42 Gln-61
Cat. # CN04

Upon arrival store at 4°C (desiccated)
See datasheet for storage after reconstitution

Background Information

The G-switch™ line of small G-protein tools has been developed with an emphasis on creating highly potent reagents that target endogenous Rho family proteins and pathways. In contrast to methods that rely on over-expression or knockdown of target proteins (e.g. DNA transfection of dominant negative or constitutively active Rho mutants, RNAi knockdown) , the G-switch™ reagents act rapidly on the endogenous target protein (in minutes to hours, depending on product), thereby optimizing the chance of generating a more physiologically relevant response. The G-switch™ product line includes reagents that directly and indirectly modulate Rho family signal transduction, thereby offering a wide range of mechanistic tools to study these critical cellular functions. See Cytoskeleton's web site for the latest G-switch™ information.

The active site of CN04 is based on the catalytic domain of the bacterial cytotoxic necrotizing factor (CNF) toxins. The catalytic domain is covalently attached to a proprietary cell penetrating moiety. CN04 directly activates Rho GTPase isoforms by deamidating glutamine-63 of Rho and glutamine-61 of Rac and Cdc42 in their respective Switch II regions (1,2). This modification converts glutamine-63 to glutamate, which blocks intrinsic and GAP-stimulated GTPase activity, resulting in constitutively active endogenous Rho, Rac and Cdc42 (3). CN04 robustly increases the level of GTP-bound RhoA, Rac1 and Cdc42 within 2-4 h after addition to the culture medium. CN04 can be used when a direct activator of Rho family proteins is required rather than a classic indirect activator (e.g., LPA, EGF, Bradykinin and Sphingosine-1-phosphate) that concomitantly activate other signaling pathways (e.g., Ras, PI3K and PLC).

Material

CN04 has been produced in a bacterial expression system. The recombinant protein has a molecular weight of 118 kDa and contains six histidine residues at its amino terminus (His tag). It has been purified to >80% purity using immobilized metal affinity chromatography (Fig. 1). Supplied as an off-white lyophilized solid, each vial contains 20 µg of CN04 protein. The material has been shown to be active in a biological assay for Rho/Rac/Cdc42 activation (see below). A protein containing a single point mutation that inactivates the catalytic domain of CN04 showed zero activity in a biological assay for Rho/Rac/Cdc42 activation (data not shown).

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. The lyophilized pellets of CN04 may appear loose and lightly packed, but this does not affect the quality or performance of CN04. For reconstitution, briefly centrifuge to collect the product at the bottom of the tube and resuspend each vial in 200 µl of sterile water, place on ice for 10 minutes prior to mixing, and mix by gently pipetting up and down to yield a concentration of 0.1 µg/µl. Prior to use, the reconstituted CN04 should be further diluted with serum free growth medium to a concentration between 0.25 and 5 µg/ml (to be determined by user, also see Figure 2 & Table 1). Typically, a 1 µg/ml concentration produces robust activation of RhoA, Rac1 and Cdc42 in multiple cell types within 3 h. Reconstituted activator can be snap frozen in liquid nitrogen and stored at –70°C for up to 6 months.

Biological Activity Assay

CN04 (1 µg/ml / equivalent to 10 µl/ml) was shown to induce a 5 – 6 fold activation of RhoA and Rac1 and a 2 fold activation of Cdc42 in Swiss 3T3 cells after a 2h incubation at 37°C (Fig. 2 & 3). After this treatment, cell morphology exhibited multiple stress fiber formation and some ruffling, consistent with activation of Rho family proteins (Fig. 4). Each member of the Rho family may be activated to a different extent by CN04 treatment in a given cell type. Table 1 gives some examples of Rho family responses to CN04.

Activity Assay Method: Swiss 3T3 cell activation

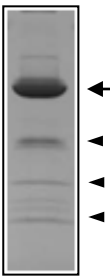
1. Grow Swiss 3T3 cells at 37°C / 5% CO_2 to 30% confluency in two 10 cm^2 dishes containing 10 ml DMEM / 10% fetal bovine serum (FBS).
2. Serum starve cells by changing media to DMEM / 1% FBS for 24 h and then transferring to DMEM / 0% FBS for 24 h.
3. Briefly spin tube of CN04 to collect contents on the bottom of the tube.
4. Reconstitute CN04 with 200 µl of sterile water (0.1 µg/µl) - See Storage and Reconstitution section above.
5. Dilute CN04 to 1 µg/ml with warm DMEM (10 µl CN04 stock per 1 ml of DMEM).
6. Aspirate medium from both dishes of cultured cells and transfer CN04 containing medium onto one dish.
7. Transfer warm DMEM lacking CN04 to the second dish. This is the control and represents unstimulated cells.
8. Incubate for 2-3 h at 37°C and 5% CO_2. Assay RhoA, Rac1 and Cdc42 activity by G-LISA® analysis (Cat. # BK124, BK128 and BK127 respectively; Figs 2 & 3) or cell morphology (Cat. # PHDG1; Fig. 4)

Table 1. Suggested Conditions for Rho Family activation

Cell Type	Cell Line	Phenotype at 1 µg/ml CN04		
		RhoA	Rac1	Cdc42
Fibroblast	Swiss 3T3	Strong	Strong	Moderate
Epithelial	HeLa	Weak	Strong	Strong
Endothelial	HUVEC*	Moderate	Moderate	Weak
	HUVEC* 2.5 – 5 µg/ml	Moderate	Strong	Moderate

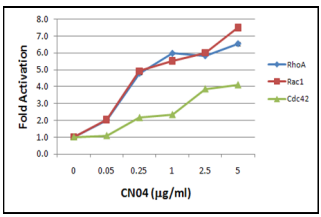
Legend: The indicated cell lines were subjected to RhoA, Rac1 and Cdc42 G-LISA® activation assays with CN04 in serum free media. Resulting phenotypes shown in Table 1 are defined by the % increase in activation (as determined by G-LISA®) for a given small G-protein above a serum starved basal level after 3h incubation; Strong >300%; Moderate >150%, Weak <120%.

Figure 1. Purity analysis of CN04 protein



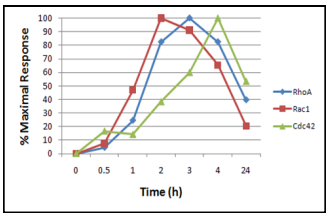
Legend: A 10 µg sample of CN04 protein was separated by electrophoresis in a 4-20% SDS-PAGE system and stained with Coomassie Blue. Densitometric analysis determined the protein to be >80% pure. Large arrow indicates CN04, faint bands under CN04 represent minor degradation products (small arrowheads).

Figure 2. Dose dependent activation of RhoA, Rac1 & Cdc42 in Swiss 3T3 cells



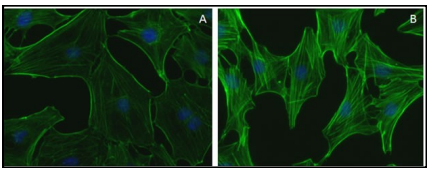
Legend: Swiss 3T3 fibroblasts were grown for 2 days in DMEM plus 10% FBS and serum starved for 24 h in media containing 1% FBS followed by 24 h in serum free media. Cells were treated with 0.05 to 5 µg/ml CN04 for 2 h and cell lysates were prepared using G-LISA® lysis buffer (Part# GL36) and were subjected to G-LISA® activation assays for RhoA (Cat.# BK124), Rac1 (Cat.# BK128) and Cdc42 (Cat.# BK127). Fold activation for each small G-protein was calculated from background-subtracted data.

Figure 3. Time dependent activation of RhoA, Rac1 & Cdc42



Legend: Swiss 3T3 fibroblasts were grown as described in Fig. 2. Cells were treated with 1 µg/ml CN04 for the indicated times and cell lysates were prepared using G-LISA® lysis buffer (Part# GL36) and were subjected to G-LISA® activation assays for RhoA (Cat.# BK124), Rac1 (Cat.# BK128) and Cdc42 (Cat.# BK127). Data were normalized to the maximal activation level for each small G-protein to clearly show the time course of activation.

Figure 4. Actin cytoskeleton morphology changes induced by CN04 treatment of Swiss 3T3 cells



Legend: Swiss 3T3 fibroblasts were grown as described in Fig.2. Cells were treated with a buffer control (A) or 1 µg/ml CN04 for 2 h at 37°C/5% CO_2. Cells were then fixed, stained with Acti-stain™ 488 phalloidin (Cat.# PHDG1), and visualized by fluorescence microscopy. Images were taken at a magnification of 40x. The control cells (A) exhibited a morphology consistent with low activity of Rho family proteins, including very few stress fibers. Treatment with CN04 (B) resulted in the development of abundant stress fibers and edge ruffling, consistent with Rho family activation.

Product Uses

- Control for Rho, Rac and Cdc42 pathway activation
- Study the effects of Rho family small G-protein activation on other signaling pathways
- Study the cell-type specific cross-talk between signaling pathways for Rho, Rac and Cdc42
- Study the effects of Rho family small G-protein activation on the re-arrangement of the actin cytoskeleton

References

1. Lerm M., et al. 1999. Deamidation of Cdc42 and Rac by Escherichia coli cytotoxic necrotizing factor 1: activation of c-Jun N-terminal kinase in HeLa cells. *Infection and immunity*. 67, 496-503.
2. Schmidt G., et al. 1997. Gln 63 of Rho is deamidated by Escherichia coli cytotoxic necrotizing factor-1. *Nature*. 387, 725-729.
3. Flatau G., et al. 1997. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature*. 387, 729-733.

Product Citations / Related Products

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