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Distinct kinetics of (H/K/N)Ras glucosylation and Rac1 glucosylation catalysed by *Clostridium sordellii* lethal toxin

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ABSTRACT

Mono-glucosylation of (H/K/N)Ras by *Clostridium sordellii* lethal toxin (TcsL) blocks critical survival signaling pathways, resulting in apoptotic cell death. One yet unsolved problem in studies on TcsL is the lack of a method allowing the specific detection of (H/K/N)Ras glucosylation. In this study, we identify the Ras(Mab 27H5) antibody as a glucosylation-sensitive antibody capable for the immunoblot detection of (H/K/N)Ras glucosylation in TcsL-treated cells. Alternative Ras antibodies including the K-Ras(Mab F234) antibody or the v-H-Ras(Mab Y13-159) antibody recognize Ras proteins regardless of glucosylation. (H/K)Ras are further shown to be more efficaciously glucosylated by TcsL than Rac1 in rat basophilic leukemia cells as well as in a cell-free system.

Structured summary:
MINT-7261742: *TcsL* (uniprotkb:Q46342) *enzymaticly reacts* (MI:0414) *H-RAS* (uniprotkb:P01112) by *enzymatic studies* (MI:0415)
MINT-7261729: *TcsL* (uniprotkb:Q46342) *enzymaticly reacts* (MI:0414) *Rac1* (uniprotkb:P63000) by *enzymatic studies* (MI:0415)
MINT-7261772: *TcsL* (uniprotkb:Q46342) *enzymaticly reacts* (MI:0414) *K-RAS* (uniprotkb:P01116) by *enzymatic studies* (MI:0415)
MINT-7261784: *TcsL* (uniprotkb:Q46342) *enzymaticly reacts* (MI:0414) *N-RAS* (uniprotkb:P01111) by *enzymatic studies* (MI:0415)

1. Introduction

Lethal toxin (TcsL) and hemorrhagic toxin (TcsH) are regarded as the major virulence factors of *Clostridium sordellii*-associated diseases, including enteritis and enterotoxaemia in cattle and sheep and myo-necrosis and gangrene in humans [1,2]. TcsL and TcsH exert their biological activity through mono-glucosylation of Rho and Ras proteins at a pivotal threonine within the effector loop (Thr-37 in RhoA, Thr-35 in Rac/Cdc42 or (H/K/N)Ras) [3,4]. Mono-glucosylation uncouples Rho/Ras proteins from their regulatory proteins and effector proteins and renders them functional inactive [5–8]. While TcsH specifically glucosylates Rho proteins, TcsL glucosylates Rac as well as the Ras family proteins (H/K/N/R)Ras, Rap(1/2), and Ral [9–12]. Treatment of cultured cells with TcsL results in actin re-organization ("cytopathic effect"), that is likely based on Rac1 glucosylation [13]. TcsL further causes apoptotic cell death ("cytotoxic effect"), likely based on the inhibition of (H/K/N)Ras/phosphatidylinositide 3′-OH kinase (PI3 K)/Akt survival signaling [10,14,15].

We recently identified the Rac1(Mab 102) antibody as a glucosylation-sensitive antibody allowing the immunoblot detection of Rac1 glucosylation [10,16,17]. This non-radioactive method has been appreciated by many researchers in the field [18,19]. One yet unsolved problem in studies on TcsL is the lack of a method allowing the detection of (H/K/N)Ras glucosylation. The glucosylation of cellular Rho proteins can be tracked by either sequential [^{32}P]ADP-ribosylation or sequential [^{14}C]glucosylation [16]. The levels of endogenous (H/K/N)Ras, however, are low (compared to that of Rho proteins) in most cell lines, for which reason [^{14}C]glucosylated, endogenous (H/K/N)Ras is only detected upon immunoprecipitation [3]. In this study, we characterize the Ras(Mab 27H5) antibody as a glucosylation-sensitive antibody capable for the immunoblot detection of (H/K/N)Ras glucosylation in TcsL-treated cells. We further show that (H/K)Ras are more efficaciously glucosylated than Rac1 in TcsL-treated rat basophilic leukemia cells.

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2. Materials and methods

2.1. Materials

Commercially obtained reagents: UDP-[^{14}C]glucose (Biotrend); antibodies: K-Ras(Mab F234, SantaCruz); Ras(Mab 27H5, Cell Signaling); v-H-Ras(Mab Y13-259, Calbiochem); Rac1(Mab 102; Transduction Lab); Rac1(Mab 23A8, Upstate); RhoA(Mab 26C4, SantaCruz); RhoB (Mab C-5, SantaCruz); GST (Pab Z-5; SantaCruz), β-actin (Mab AC-40, Sigma), and horseradish peroxidase conjugated secondary antibodies rabbit/mouse (Rockland).

2.2. Methods

2.2.1. Toxin purification

TcdBF was purified from *C. difficile* serotype F strain 1470 and TcsL from *C. sordellii* strain 6018 as described [20]. Briefly, the toxins were precipitated from culture supernatants of the respective strain by 70% ammonium sulfate saturation. Subsequently, the toxins were dialyzed and loaded onto MonoQ columns. After dialysis against buffer (50 mM TRIS, pH 7.4, 15 mM NaCl), the toxins were ready for use.

2.2.2. Protein purification

GST-tagged Rho and Ras proteins were expressed in *Escherichia coli* using the pGEX-2T vector system and affinity purified using Glutathion-Sepharose Beads (AP Biotech).

2.2.3. Cell culture

Rat basophilic leukemia (RBL-2H3) cells that express (H/K)Ras but not N-Ras were grown as adherent monolayers on tissue culture flasks in minimum essential medium plus Earle's salts (MEM plus Earle's; Biochrom) supplemented with 15% heat-inactivated fetal calf serum, 100 μg/mL penicillin, 100 U/mL streptomycin, and 1 mM sodium pyruvate. Cells were maintained in 5% CO_2 at 37 °C. Upon confluence, cells were passaged. For all experiments, subconfluent cells in 3 cm dishes were treated with TcdBF or TcsL as indicated for 4 h.

2.2.4. Sequential [^{14}C]glucosylation

Cells were washed and lysed in a buffer containing NaCl (150 mM), Tris (50 mM, pH 7.2), $MgCl_2$ (5 mM), PMSF (1 mM), and NP40 (1%). After sonication, the soluble fraction was prepared by centrifugation and incubated with TcdBF or TcsL, respectively, in the presence of UDP-[^{14}C]glucose for 30′ at 37 °C. The reaction was terminated by addition of Laemmli sample buffer. Subsequently, the samples were separated by SDS PAGE and subjected to PhosphoImager analysis.

2.2.5. Western blot analysis

Complete lysate proteins were separated using SDS–PAGE and subsequently transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) non-fat dried milk in 50 mM Tris, pH 7.2, 150 mM NaCl, 0.05% TWEEN 20 for 60 min; incubation with primary antibody was conducted over night at 4 °C. After treatment of the membrane with secondary antibody (1 h), proteins were detected with ECL Femto (Pierce). For immunoblot detection of Ras and Rac1 from cell lysates the following antibody concentrations were applied: Ras(Mab 27H5) 0.5 μg/ml, K-Ras(-Mab F234) 0.5 μg/ml, Rac1(Mab 102) 0.25 μg/ml, Rac1(Mab 23A8) 0.3 μg/ml. For the immunoblot detection of GST-tagged Ras proteins the following antibody concentrations were applied: Ras(Mab 27H5) 5 ng/ml, K-Ras(Mab F234) 20 ng/ml, v-H-Ras(Mab Y13-259) 50 ng/ml, GST(Pab Z-5) 5 ng/ml.

2.2.6. Glucosylation reaction

Recombinant (H/K/N)Ras and Rac1 (2 μg) were incubated with or without TcsL in glucosylation buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 100 mM KCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 100 μg/ml BSA, 10 μM UDP-[^{14}C]glucose, 10 μM UDP-glucose) at 37 °C for 30 min or the indicated periods. The reaction was terminated by addition of Laemmli sample buffer. Proteins were separated by SDS–PAGE and analyzed by immunoblotting and phosphorImaging (Cyclone, Packard).

2.2.7. Statistical analysis

The experimental data were all determined to be statistically accurate to a standard deviation of not greater than 10%. The kinetic parameters were determined from Eadie–Hofstee plots of initial rates of reaction as a function of substrate protein concentration.

3. Results

3.1. Effect of glucosylation at Thr-35 on the recognition of Ras isoforms by Ras antibodies

Several anti-Ras antibodies were screened for their reactivity with GST-tagged versions of H-Ras, K-Ras, and N-Ras using immunoblot analysis (Fig. 1). The Ras(Mab27H5), the v-H-Ras(Y13-259), and the GST(Z-5) antibodies equally detected either GST-tagged H-Ras, K-Ras, or N-Ras (Fig. 1). In contrast, the K-Ras(Mab F234) antibody specifically recognized K-Ras but recognized neither H-Ras nor N-Ras (Fig. 1).

The antibodies were next screened for their ability to detect glucosylated (H/K/N)Ras. Therefore, GST-tagged versions of either K-Ras, H-Ras, or N-Ras were [^{14}C]glucosylated with TcsL or left non-glucosylated. [^{14}C]glucosylation of either K-Ras (Fig. 2A), H-Ras (Fig. 2B), or N-Ras (Fig. 2C) was detected by autoradiography. GST-K-Ras was detected by either the GST(Pab Z-5) antibody, the v-H-Ras(Mab Y13-259) antibody, or the K-Ras(Mab F234) antibody regardless of glucosylation (Fig. 2A). In contrast, detection of glucosylated K-Ras (Fig. 2A), glucosylated H-Ras (Fig. 2B), and glucosylated N-Ras (Fig. 2C) by the Ras(Mab 27H5) antibody was strongly reduced. The GST(Z-5) antibody or the v-H-Ras(Mab Y13-259) antibody detected H-Ras (Fig. 2B) or N-Ras (Fig. 2C) regardless of glucosylation. Signal intensities of immunoblot detection of either K-Ras, H-Ras, or N-Ras by either the glucosylation-sensitive Ras(Mab 27H5) or a selected antibody, that detected either Ras protein regardless of glucosylation, was densitometrically quantified (Fig. 2D). Detection of either H-Ras, K-Ras, or N-Ras by the Ras(Mab 27H5) antibody (not by the other applied antibodies) was thus sensitive to TcsL-catalysed glucosylation at Thr-35.

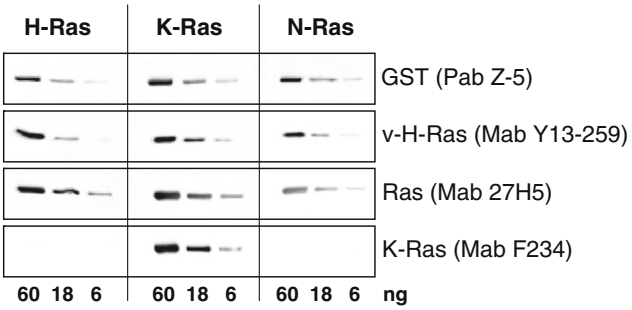

Fig. 1. Detection of (H/K/N)Ras by Ras antibodies. Decreasing amounts (60 ng, 18 ng, 6 ng) of either GST-tagged H-Ras, K-Ras, or N-Ras were analyzed for immunoblot detection using the indicated antibodies. The displayed immunoblots are representative from three independent experiments.