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The Ras-Related Protein Ral Is Monoglucosylated by *Clostridium sordellii* Lethal Toxin[1]

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Clostridium sordellii lethal toxin (LT), a cytotoxin which causes preferential destruction of the actin cytoskeleton, has been recently identified as glucosyltransferase to modify the low molecular mass GTPases Rac, Ras and Rap. We report here on LT produced by *C. sordellii* strain 6018 which glucosylates in addition to Rac, Ras and Rap the Ral protein. LT from strain VPI9048 however does not glucosylate Ral. Besides recombinant Ral, cellular Ral is also substrate. In the GDP-bound form, Ral is a superior substrate to the GTP form. Acceptor amino acid for glucose is threonine-46 which is equivalent to threonine-35 in H-Ras located in the effector region. The Ral-glucosylating toxin is a novel isoform of Ras-modifying clostridial cytotoxins.

Clostridium sordellii lethal toxin (LT) acts on cultured cell lines cytotoxically by inducing preferential destruction of the actin cytoskeleton (1-3). Recently, LT has been identified as glucosyltransferase to modify Ras, Rap and Rac low molecular mass GTPases (4,5). Glucosylation of Ras resulted in functional inactivation to block the mitogen-activated protein (MAP) kinase signal pathway. Acceptor amino acid is threonine-35 which is located in the effector region of Ras. This location of the glucose moiety seems to be responsible for the blockade of effector coupling. The hydroxyl group of threonine-35 participates in GTP-binding via coordination of the Mg^{2+} ion. In the GDP-bound form, this hydroxyl group changes its orientation and is exposed to the surface of the molecule (6). Therefore, the GDP-form of Ras is the preferential substrate for glucosylation.

LT belongs to the family of large clostridial cytotoxins which also encompasses the *Clostridium difficile* toxin A and toxin B. The large clostridial cytotoxins are single-chained toxins of a molecular mass of 250 to 300 kDa exhibiting a homology of 35 to 90% to each other. Furthermore, they seem to act by a common mode of action to cause depolymerization of the actin cytoskeleton. Also *Clostridium difficile* toxin A and toxin B are glucosyltransferases. In contrast to LT, they glucosylate selectively the Rho subfamily proteins Rho, Rac and Cdc42 but not members of other subfamilies of low molecular mass GTPases (7-9). Glucosylation of threonine-37/35, which is the acceptor amino acid in Rho, Rac and Cdc42, causes inactivation of Rho resulting in depolymerization of the actin filament system (7).

The protein substrate specificity of LT which encompasses Rac, Ras and Rap were reported by two groups which used different *Clostridium sordellii* strains to purify LT, namely IP82 (5) and 6018 (4), respectively. We report here that LT from strain 6018 and strain CN6 glucosylate the Ral protein, a member of the Ras subfamily.

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Abbreviations: LT, *Clostridium sordellii* lethal toxin; LT^{VPI}, LT from *Clostridium sordellii* strain VPI9048; LT^{6018}, LT from strain 6018.

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MATERIALS AND METHODS

Materials. ^{14}C-labeled UDP-glucose was obtained from Dupont NEN (Dreieich, Germany). All other reagents were of analytical grade and purchased from commercial sources. Preparation of lysates from rat brain homogenates: rat brains were homogenized with 5 volumes of lysis buffer (1 mM $MgCl_2$, 40 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 80 μg/ml benzamidine, 50 mM HEPES, pH 7.4), followed by centrifugation for 10 min $\times$ 2,000 g. The supernatant was used as lysate.

Purification of Clostridium sordellii lethal toxin (LT). Purification of LT from *Clostridium sordellii* strain VPI9048 and 6018 were performed after the procedure according to (10). Briefly, the culture was grown in a dialysis bag under microaerophilic conditions at 37°C for 72 h. The ammonium sulfate precipitate was extracted with 50 mM Tris-HCl (pH 7.5), dialysed against 2 $\times$ 1 l of the same buffer and then applied onto a MonoQ column (HR 10/10; Pharmacia, Freiburg), previously equilibrated with 50 mM Tris-HCl (pH 7.5). The bound proteins were eluted by using a linear gradient from 50 mM to 700 mM NaCl. LT (from both strains) eluted at 500 mM NaCl.

Preparation of recombinant GTP-binding proteins. Rac1, Cdc42, H-Ras, Rho, Ral and Ral^{T46A} were prepared from their fusion proteins (e.g. RhoA-glutathione S-transferase) as described (11). Ral^{T46A} was generated by PCR-based *in vitro* mutagenesis. Glutathione S-transferase (GST)-fusion proteins from the *E. coli* expression vector pGEX-2T were isolated by affinity purification with glutathione-Sepharose (Pharmacia, Germany) followed by release of the GTP-binding proteins from the GST-fusion protein by thrombin treatment (100 μg/ml for 30 min at 22°C). Thrombin was removed by binding to benzamidine-Sepharose (Pharmacia, Freiburg) and the GTP-binding proteins were concentrated with Centricon (Amicon).

Glucosylation reaction. Recombinant GTP-binding proteins (50 μg/ml) were incubated with LT (4 μg/ml) in a buffer containing 20 μM UDP-[^{14}C]glucose, 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, 100 μg/ml bovine serum albumin and 50 mM HEPES (pH 7.4) for the indicated times at 37°C. Lysates from rat brain homogenates were incubated in the presence of 20 μM UDP-[^{14}C]glucose with LT (4 μg/ml) for 45 min at 37°C. The reactions were terminated by addition of Laemmli sample buffer. Loading of Ral with guanyl nucleotides were performed by incubating Ral (50 μg/ml) with 2.5 mM of either GDP or GTP[S] for 60 min at 20°C.

Gel electrophoresis. Proteins were dissolved in sample buffer and subjected to 12.5% SDS-PAGE (12) followed by analysis using the PhosphorImager SF from Molecular Dynamics.

Immunoblot. Immunoblotting was performed according to Towbin et al. (13) with anti-Ral monoclonal antibody from Oncogene Science (Uniondale, NY), and anti-Rho, anti-Cdc42 and anti-Rac2 from Santa Cruz (California, USA). Visualization was performed with the Amersham enhanced chemiluminescence system (Amersham Corp., Arlington Heights).

RESULTS AND DISCUSSION

Incubation of rat brain lysates with LT from *C. sordellii* strain VPI9048 (LT^{VPI}) and LT from strain 6018 (LT^{6018}), respectively, in the presence of UDP-[^{14}C]glucose resulted in a different labeling pattern. Whereas LT^{VPI} catalyzed glucosylation of a protein band in the molecular mass range of 22 kDa, LT^{6018} caused the labeling of a double band of 22 and 27 kDa (Fig. 1A). For comparison the lysates were incubated with toxin B from *C. difficile* which [^{14}C]glucosylates Rho (upper band) and Rac/Cdc42 (lower band) (8). To identify the novel substrate protein of LT^{6018}, several recombinant low molecular mass GTPases were tested to serve as substrate for LT^{6018} and for LT^{VPI}, respectively. LT^{6018} catalyzed glucosylation of Rac and H-Ras as has been reported (4) (Fig. 1B). Furthermore, we found that Rap1 is glucosylated as has been found for strain IP82 (5). However, LT^{6018} was able to glucosylate Ral and to a minor extent (about 5%) Cdc42 in addition to Rac, Ras and Rap. Recently, we reported that LT^{6018} did not glucosylate Cdc42 (4). The observation that Cdc42 is now a minor substrate for LT^{6018} is most likely based on the different preparation procedure of the toxin which yielded in more active toxin. Interestingly, LT^{VPI}, a novel isoform, showed a different substrate specificity (Fig. 1B). Rac1, Cdc42, H-Ras and Rap1 were substrate proteins but not Ral. In contrast to LT^{VPI}, the Ras subfamily proteins H-Ras and Rap1 were favoured substrates for LT^{6018}. Prototypes of other subfamilies of the superfamily of low molecular mass GTPases were not glucosylated. LT prepared from *C. sordellii* strain CN6 exhibited the same protein substrate specificity as LT^{6018} (not shown).

Ral has a molecular mass of 23,6 kDa as deduced from the amino acid sequence, but exhibits apparently 27 kDa on SDS-PAGE (14) and could be the novel target protein. Immunoblot

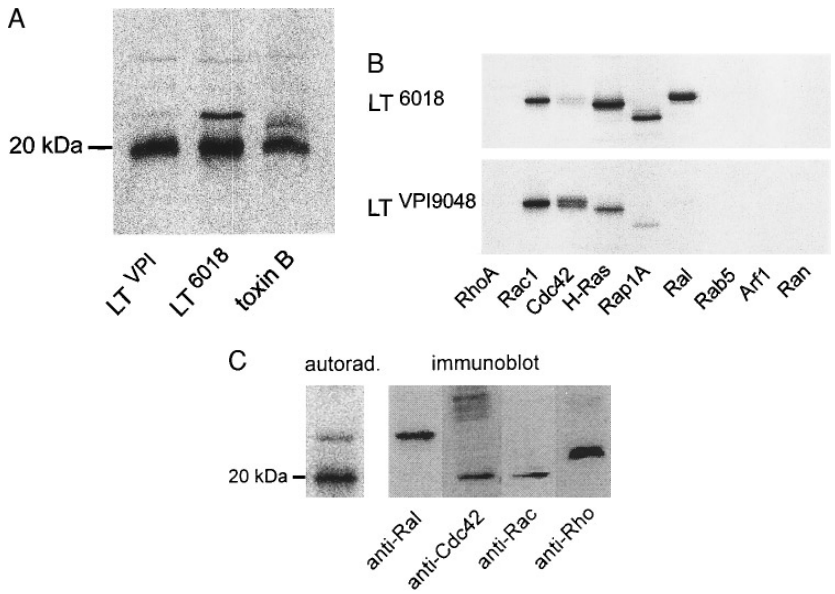

FIG. 1. Protein substrate specificity. **A.** Rat brain lysates were incubated with LT^{VPI}, LT^{6018} and toxin B in the presence of UDP-[^{14}C]glucose for 30 min at 37°C. **B.** Recombinant low molecular mass GTPases (RhoA, Rac1, Cdc42, H-Ras, Rap1A, Ral, Rab5, Arf1, Ran; each 50 μg/ml, dissolved in a buffer containing 1 mM $MgCl_2$, 50 mM HEPES (pH 7.5) and 100 μg/ml bovine serum albumin) were incubated with the LTs (4 μg/ml plus 0.1 mM $MnCl_2$) and ^{14}C-labeled UDP-glucose (30 μM) for 30 min at 37°C. PhosphorImager data (A,B) of the 12.5% SDS-gel electrophoresis are shown. **C.** Rat brain lysate ^{14}C-glucosylated with LT^{6018} was electroblotted and probed with anti-Ral, anti-Cdc42, anti-Rac2 and anti-Rho.

analysis of rat brain lysate with anti-Ral, anti-Rho and anti-Rac revealed that the [^{14}C]-glucosylated upper protein band was recognized by anti-Ral (Fig. 1C). Thus, LT^{6018} represents a novel isoform of LT glucosyltransferases which modifies Rac and the Ras subtype proteins Ras, Rap and Ral.

Threonine-35 (Thr-35) in Rac (4) as well as in H-Ras (5) has been identified as acceptor amino acid for LT-catalyzed glucosylation. Thus, we attempted to identify the acceptor in Ral by exchanging the putative acceptor threonine-46 (Thr-46) to alanine by mutagenesis. Thr-46 is equivalent to Thr-35 in Ras and Rac. As illustrated in Fig. 2, Ral^{T46A} is not substrate for LT^{6018} indicating that Thr-46 is the exclusive acceptor amino acid and that there are no additional acceptors in Ral. Because Thr-46 participates in GTP-binding we tested the guanyl nucleotide dependence of the glucosylation reaction. The GDP-bound Ral is a better substrate

FIG. 2. Acceptor amino acid of glucosylation. WT Ral and mutant RalT46A (50 μg/ml, dissolved in 1 mM $MgCl_2$/50 mM HEPES pH 7.5/100 μg/ml bovine serum albumin) were ^{14}C-glucosylated with LT^{6018} (4 μg/ml plus 0.1 mM $MnCl_2$) for 60 min at 37°C. PhosphorImager data of the 12.5% SDS-gel electrophoresis are shown.

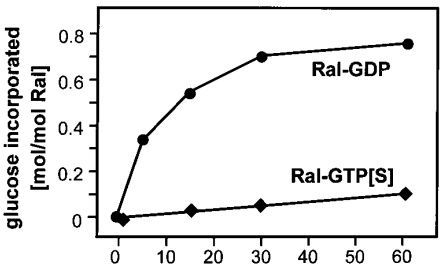

FIG. 3. Guanyl nucleotide dependence of the glucosylation reaction. WT Ral was incubated with GDP and GTP[S] (each 2.5 mM) for 1 h at 20°C followed by ^{14}C-glucosylation with LT^{6018} (4 μg/ml plus 0.1 mM $MnCl_2$) for the indicated times at 37°C.

for LT^{6018} than the GTP[S]-bound form as shown in Fig. 3 which gives the time dependence of the glucosylation reaction. Binding of the nonhydrolyzable GTP-analogue GTP[S] almost completely blocked glucosylation. In the GDP-bound form, not more than 0.8 mol of glucose was incorporated per mol of Ral consistent with mono-glucosylation. Applying the crystal structure of Ras to the Ral protein, Thr-46 in Ral (corresponding to Thr-35 in Ras) is located in the effector domain and is involved in GTP-binding through coordination of Mg^{2+}. In the GDP-bound form the hydroxyl group of Thr-46 is directed to the surface of the molecule to be now accessable for glucosylation. The data are consistent with this model and are in agreement with those reported for *C. difficile* toxin A and toxin B which selectively catalyze mono-glucosylation of Thr-37 in Rho (7,8). Glucosylation occurs in the effector region of the GTPases and a hydrophilic moiety herein most likely causes inhibition in GTPase-effector coupling thereby blocking the signal cascade.

Whereas Ras plays a crucial role in cell transformation and differentiation, it seems that Rap is a physiological antagonist of Ras (15). Ral functions are not so well documented as those of Ras but there is evidence that Ral is a down-stream target of Ras and Rap (16-18). Recently, it has been reported that RalA interacts with phospholipase D and various GAPs for Rac and Cdc42 (19-21). Comparison of the cellular effects of LT^{VPI} with those of LT^{6018} is a valuable cell biological approach to get more insight into the functions of Ral.

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REFERENCES

1. Baldacini, O., Girardot, R., Green, G. A., Rihn, B., and Monteil, H. (1992) *Toxicon* **30,** 129–140.
2. Ciesielski-Treska, J., Ulrich, G., Baldacini, O., Monteil, H., and Aunis, D. (1991) *Eur. J. Cell Biol.* **56,** 68–78.
3. Popoff, M. R. (1987) *Infect. Immun.* **55,** 35–43.
4. Just, I., Selzer, J., Hofmann, F., Green, G. A., and Aktories, K. (1996) *J. Biol. Chem.* **271,** 10149–10153.
5. Popoff, M. R., Chaves, O. E., Lemichez, E., Von Eichel-Streiber, C., von Thelestam, M., Chardin, P., Cussac, D., Chavrier, P., Flatau, G., Giry, M., Gunzburg, J., and Boquet, P. (1996) *J. Biol. Chem.* **271,** 10217–10224.
6. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) *EMBO J.* **9,** 2351–2359.
7. Just, I., Selzer, J., Wilm, M., Von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *Nature* **375,** 500–503.
8. Just, I., Wilm, M., Selzer, J., Rex, G., Von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *J. Biol. Chem.* **270,** 13932–13936.
9. Aktories, K., and Just, I. (1995) *Trends in Cell Biology* **5,** 441–443.

10. Just, I., Selzer, J., Hofmann, F., and Aktories, K. (1997) *in* Microbial Toxins (Aktories, K., Ed.), Chapman & Hall, Weinheim, in press.
11. Just, I., Fritz, G., Aktories, K., Giry, M., Popoff, M. R., Boquet, P., Hegenbarth, S., and Von Eichel-Streiber, C. (1994) *J. Biol. Chem.* **269,** 10706–10712.
12. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
13. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
14. Bhullar, R. P., Chardin, P., and Haslam, R. J. (1990) *FEBS Lett.* **260,** 48–52.
15. Bokoch, G. M. (1993) *Biochem. J.* **289,** 17–24.
16. Macara, I. G., Lounsbury, K. M., Richards, S. A., McKiernan, C., and Bar-Sagi, D. (1996) *FASEB J.* **10,** 625–630.
17. Herrmann, C., Horn, G., Spaargaren, M., and Wittinghofer, A. (1996) *J. Biol. Chem.* **271,** 6794–6800.
18. Ikeda, M., Koyama, S., Okazaki, M., Dohi, K., and Kikuchi, A. (1995) *FEBS Lett.* **375,** 37–40.
19. Jiang, H., Luo, J.-Q., Urano, T., Frankel, P., Lu, Z., Foster, D. A., and Feig, L. A. (1995) *Nature* **378,** 409–412.
20. Park, S. H., and Weinberg, R. A. (1995) *Oncogene* **11,** 2349–2355.
21. Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gacon, G., and Camonis, J. H. (1995) *J. Biol. Chem.* **270,** 22473–22477.