Morphological alterations of *Xenopus* oocytes induced by valine-14 p21^{rho} depend on isoprenylation and are inhibited by *Clostridium botulinum* C3 ADP-ribosyltransferase

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Microinjection of the constitutively active recombinant Val-14 p21rho A into *Xenopus* oocytes induced dramatic morphological changes with redistribution of pigments from the animal pole resulting in spotted oocytes. The effects induced by Val-14 p21$^{rho\ A}$ were regulated by progesterone in a dose-dependent manner whereas prior ADP-ribosylation of the rho protein blocked its activity. About 30 min after microinjection, p21 rho was associated with the plasma membrane. The membrane association of p21^{rho} and its biological activity were inhibited by lovastatin, an inhibitor of the 3-hydroxy-3-methylglutaryl coenzyme A reductase. The findings suggest that membrane attachment and biological activity of p21^{rho} depend on isoprenylation of the GTP-binding protein.

Clostridium botulinum C3 ADP-ribosyltransferase; *Xenopus* oocyte; GTP-binding protein; Rho; Cytoskeleton; Lovastatin

1. INTRODUCTION

A still increasing number of low molecular mass GTP-binding proteins with M_r values of about 20 000 have been recognized in recent years. The best-studied is the ras protein family, which has been the focus of intense research for several years because mutant alleles are frequently observed in some human tumors [1]. Other members of this family that are related to ras are the ral [2], rap [3], rab [4] rho [5], and rac [6] gene families. The encoded proteins all share the ability to bind GTP and GDP with high affinity. They are apparently active in the GTP-bound form and inactive after hydrolysis of GTP to GDP caused by an intrinsic GTPase activity of the proteins [1].

Many GTP-binding proteins are known to be substrates for bacterial ADP-ribosyltransferases and the enzymes have been useful tools in analysing the functions of these proteins. Whereas G-proteins (G_s, G_i, G_o, G_t) are ADP-ribosylated by cholera and pertussis toxins [7], low molecular weight GTP-binding proteins are not substrates for these toxins. Recently, however, the rho A, B, C [8–11], and rac 1, 2 [6] proteins have been shown to be substrates for the ADP-ribosyltransferase C3 from *Clostridium botulinum* [12–15]. So far, the cellular functions of the rho and rac proteins and the physiological consequences of their ADP-ribosylation are, however, largely obscure.

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We have attempted to analyse the function of the rho protein using a *Xenopus* oocyte system. Fully grown *Xenopus* oocytes are physiologically arrested in the prophase of the first meiotic division. Progesterone and insulin induce the so-called maturation of oocytes with progression through the meiotic divisions and arrest at metaphase II. Low molecular weight GTP-binding proteins apparently participate in the regulation of oocyte maturation. Microinjection of ras proteins into oocytes for example can induce maturation [16] whereas monoclonal anti-ras antibody was shown to inhibit insulin-induced oocyte maturation [17]. Furthermore, microinjection of *C. botulinum* ADP-ribosyltransferase C3 into oocytes reportedly accelerates the progesterone-induced maturation [11]. Consequently, we now studied the effects of microinjection of recombinant rho proteins into *Xenopus* oocytes.

2. MATERIALS AND METHODS

2.1. *Materials*

Botulinum ADP-ribosyltransferase C3 was purified as described [14]. Normal and Val-14 rho A cDNAs containing a *Hpa*I site engineered at codon 25 [18] were expressed in *E. coli* by using an expressing system as described earlier [19]. Partial purification of the rho proteins (30–50% pure) were achieved by using DEAE-Sephacel anion-exchange chromatography and G75 Sephadex gel permeation chromatography as described for ras proteins [19]. Lovastatin was a generous gift from Merck, Sharp and Dohme (Munich, FRG) [^{32}P]NAD was purchased from NEN (Dreieich, FRG). All other chemicals were analytical grade and purchased from commercial sources.

2.2. *Microinjection into* Xenopus *oocytes*

Stage VI oocytes from *Xenopus laevis* were selected after col-

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lagenase incubation of the ovarian tissue as described [20] and kept in Barth's medium (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 10 mM Hepes, pH 7.8) at 16–19°C overnight. One hour after microinjection of 50 nl of the various agents tested, progesterone was added for 1 h at the indicated concentration and, thereafter, the incubation was continued at exactly 19°C. At the indicated intervals, oocytes were scored for the morphological alterations showing the appearance of lamellar white segments in the animal pole. Usually 20 oocytes were microinjected. All experiments were repeated at least 3 times.

2.3. *Lovastatin treatment of oocytes*

Oocytes were incubated without and with 50 μM lovastatin (prepared as described [21]) for 1 h at room temperature, followed by microinjection of rho protein (about 10 ng/oocyte). Thereafter, oocytes were placed in Barth's medium containing 10 μM progesterone (for 1 h) without or with 50 μM lovastatin. At the indicated intervals the oocytes were scored for morphological changes and groups of 20 oocytes were frozen in 200 μl of a buffer containing 0.32 M sucrose and 1 mM PMSF. After thawing, the oocytes were homogenized and centrifuged for 10 min at 1000 × *g*. The supernatant was again centrifuged for 20 min at 10 000 × *g*. The pellet was resuspended in 200 μl of sucrose buffer. Pellet and supernatant (60 μl) were used in the ADP-ribosylation assay.

2.4. *ADP-ribosylation assay*

ADP-ribosylation was performed essentially as described [10]. The separated fractions of oocytes were incubated in a buffer containing 50 mM triethanolamine·HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 2 mM $MgCl_2$, 0.1 μM [^{32}P]NAD (about 0.3–0.6 μCi) and 0.15 μg C3 for 1 h at 37°C. The total volume was 100 μl. The incubation was stopped by adding 900 μl trichloroacetic acid (20%, w/v). The pellet was washed with ether, dissolved in 30 μl sample buffer and subjected to 15% gel electrophoresis according to Laemmli [22]. Gels were stained with Coomassie blue, destained and subjected to autoradiography for 72 h.

Protein concentration was determined according to Bradford [23] with ovalbumin as standard.

3. RESULTS

It has been reported that microinjection of *C. botulinum* ADP-ribosyltransferase C3 accelerates the progesterone-induced maturation of *Xenopus* oocytes [15]. We have confirmed this finding and observed that under control conditions where 50% of the oocytes underwent maturation after 16.5 h, microinjection of C3 shortened this period to about 10 h (not shown). As C3 ADP-ribosylates the GTP-binding protein rho, we were prompted to study the effect of persistently active Val-14 rho protein on the progesterone-induced maturation. Surprisingly, microinjection of Val-14 rho protein completely changed the morphology of the oocytes (Fig. 1C). While normal maturation (germinal vesicle breakdown) was indicated by the occurrence of a white spot in the animal pole (Fig. 1B), the Val-14 rho protein preparation caused a dramatic redistribution of pigments of the animal pole. At first, lamellar white segments occurred at the animal pole, which then increased in size, became confluent and finally resulted in a spotted animal pole. This phenotype was not observed after microinjection of normal rho protein. As shown in Fig. 2, the appearance of the morphological changes by Val-14 rho protein clearly depended on the presence

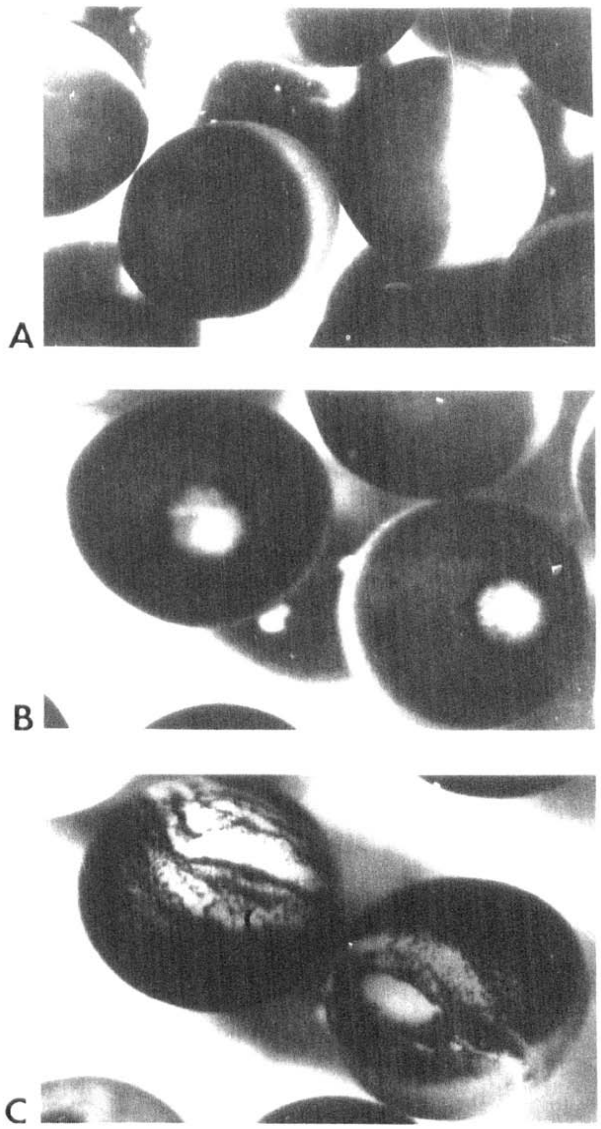

Fig. 1. Morphological alterations induced by microinjection of Val-14 $p21^{rho\ A}$. *Xenopus* oocytes were microinjected with 10 ng Val-14 $p21^{rho\ A}$ and incubated for 1 h. Thereafter, the oocytes were stimulated for 1 h in a medium containing progesterone (10 μM) and then further incubated without the hormone. (A) Control oocytes incubated without progesterone. (B) Maturated oocytes after stimulation with progesterone for 1 h and incubation for 16 h. (C) Oocytes microinjected with valine-14 $p21^{rho\ A}$ and treated with progesterone for 1 h and incubated for further 4 h.

of progesterone. Progesterone decreased the time lag and increased the rate of appearance of the rho-induced oocyte alterations. However, even at the highest concentration of progesterone, the morphological changes occurred not earlier than about 1 h after microinjection of Val-14 rho.

Fig. 3 shows that prior ADP-ribosylation of Val-14 rho by C3 inhibited its potential to cause morphological changes. Under the conditions used, only about 40% of the rho protein was modified, which might explain why

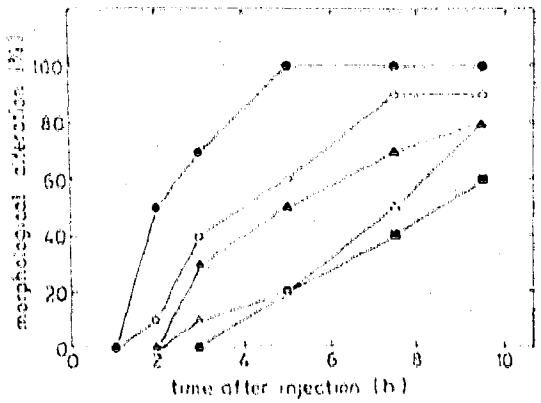

Fig. 2. Influence of progesterone on Val-14 $p21^{rho}$-induced morphological changes of oocytes. Oocytes were microinjected with Val-14 $p21^{rho}$ and then incubated for 1 h. Thereafter, the oocytes were stimulated without (■) and with progesterone (●, 10 μM; ○, 1 μM; ▲, 0.1 μM; △, 0.01 μM) for 1 h and further incubated. The appearance of the typical morphological alterations (see Fig. 2C) were scored at the indicated intervals.

Fig. 4. Localisation of the microinjected rho protein. After microinjection of Val-14 rho A protein, oocytes were incubated without (A) and with (B) lovastatin (see section 2) for the indicated periods of time. Then the soluble (S) and membrane fractions (P) of oocytes were separated and ADP-ribosylated by C3 with 0.1 μM [^{32}P]NAD. The labeled proteins were analysed by SDS-PAGE and autoradiography (shown). (con; controls, not microinjected).

ADP-ribosylation delayed the onset of the morphological changes but did not completely inhibit the Val-14 p21rho-induced effects.

It has been shown that the biological activity of the ras proteins depends on their association with the plasma membrane. We studied whether the injected rho protein was associated with the oocyte membrane. Therefore, Val-14 p21rho-microinjected oocytes were incubated for various periods of time and, thereafter, separated into soluble and membrane fractions. The presence of the rho protein was determined by [^{32}P]ADP-ribosylation with C3. Under control condition, e.g. without microinjection, C3 slightly labeled a 20 kDa protein in the membrane fraction. In contrast, after microinjection an increase in labeling of more than 10-fold occurred. As shown in Fig. 4A, 5 and 15 min after microinjection the labeled protein was observed in the soluble fraction, while at time point 30 min and later the major labeling was determined in the membrane fraction. The ADP-ribosylated protein of the supernatant migrated a little slower than that in the pellet fraction. In contrast, when the cells were treated with lovastatin, an inhibitor of the mevalonic acid pathway, no translocation of the rho protein into the membrane was detected (Fig. 4B). Fig. 5 shows that treatment of oocytes with lovastatin also inhibited the morphological changes induced by Val-14 p21rho. Fur-

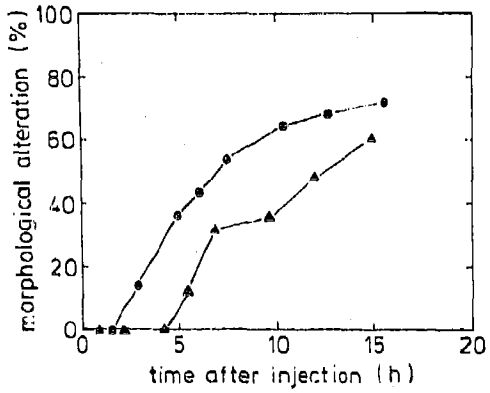

Fig. 3. Impairment of the effect of Val-14 $p21^{rho\ A}$ by ADP-ribosylation with C3. Val-14 $p21^{rho\ A}$ was ADP-ribosylated in the absence (●) and presence (▲) of NAD by C3 as described in section 2; thereafter, the pretreated proteins were microinjected and the oocytes stimulated by progesterone (10 μM) for the indicated intervals.

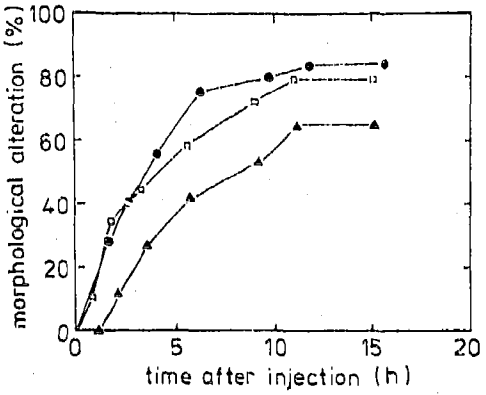

Fig. 5. Influence of lovastatin on the effects of Val-14 $p21^{rho\ A}$ protein in *Xenopus* oocytes. *Xenopus* oocytes were preincubated without (●) and with lovastatin (200 μM; ▲) and lovastatin plus mevalonic acid (0.8 mM; □) for 1 h, then 10 ng Val-14 $p21^{rho\ A}$ was microinjected and the incubation was continued for 1 h. Thereafter, the oocytes were stimulated for 1 h by progesterone (10 μM) and further incubated without the hormone. At the indicated periods of time the morphologically altered oocytes were scored.

thermore, when the lovastatin-induced inhibition of the mevalonic acid pathway was bypassed by addition of exogenous mevalonic acid, the effect of Val-14 p21rho was completely restored.

4. DISCUSSION

We report here that microinjection of persistently active Val-14 rho recombinant protein causes dramatic changes in the morphology of *Xenopus* oocytes characterized by redistribution of the pigmented half of the oocytes. Apparently, the effect of the mutant rho protein depended on the posttranslational isoprenylation of the rho protein, since lovastatin impaired the action of Val-14 rho protein on pigment redistribution of oocytes, an effect which was restored by addition of mevalonic acid. Lovastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, which blocks the formation of mevalonic acid, a precursor of isoprene residues [21]. Recently it has been demonstrated that all ras proteins are isoprenylated at cysteine-186 after proteolytic cleavage of three amino acids of the C-terminal CAAX motif [24]. This modification is apparently essential for the biological activity of the ras proteins. The rho proteins possess the same C-terminal motif [25,26]. Our findings are in agreement with the view that the rho proteins are also subject to isoprenylation. Isoprenylation increases the hydrophobicity of the protein, which most likely is a prerequisite for the association of the protein with the plasma membrane and for its full biological activity.

Prior ADP-ribosylation of Val-14 rho by C3 impaired the activity of the GTP-binding protein in *Xenopus* oocytes. A similar result was recently obtained with ADP-ribosylated rho protein microinjected into 3T3 NIH cells. C3 modifies the rho protein at asparagine-41 [27]. Deduced from the 3-dimensional model of ras [28] and provided a high structural homology between ras and rho, the ADP-ribose-acceptor in rho is located in the so-called effector region of the GTP-binding protein which might explain why ADP-ribosylation renders the protein inactive.

The effects of the Val-14 rho protein on oocytes depends on the presence of progesterone. Progesterone inhibits adenylyl cyclase, decreases the cyclic AMP levels of oocytes and causes by a sequelae of further events, which are not fully understood, the induction of oocyte maturation [29]. Since the induction of maturation by progesterone has been shown to be accelerated by botulinum ADP-ribosyltransferase C3 [15], it may be that rho proteins play a tonic regulatory role in maturation.

The precise mechanism by which the activated rho protein causes the alterations described here is unclear. However, there is evidence that rho is somehow involved in the regulation of cytoskeletal elements. For example, treatment of Vero cells or rat hepatome FAO cells with C3 causes the destruction of the microfilament network [11,30]. Furthermore, microinjection of C3 in NIH 3T3 cells induces rounding up of cells and redistribution of microfilament proteins [18]. Interestingly, injection of Val-14 rho protein into NIH 3T3 cells, causes rapid changes in cell morphology characterized by contraction of the cell body and formation of finger-like processes, a phenotype different from that induced by microinjection of C3 [18]. It is known that the internal structure of *Xenopus* oocytes is reorganized during the progesterone-induced maturation [31]. We can speculate that the role of rho in oocytes is to regulate the cytoskeletal organization. The effects we observe after microinjection of Val-14 rho may be a consequence of severe reorganization of the cytoskeletal network.

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REFERENCES

[1] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[2] Chardin, P. and Tavitian, A. (1986) EMBO J. 5, 2203–2208.
[3] Pizon, V., Chardin, P., Lerosey, I., Olofson, B. and Tavitian, A. (1988) Oncogene 3, 201–204.
[4] Touchot, N., Chardin, P. and Tavitian, A. (1987) Proc. Natl. Acad. Sci. USA 84, 8210–8214.
[5] Madaule, P. and Axel, R. (1985) Cell 41, 31–40.
[6] Didsbury, J., Weber, R.F., Bokoch, G.M., Evans, T. and Snyderman, R. (1989) J. Biol. Chem. 264, 16378–16382.
[7] Pfeuffer, T. and Helmreich, E.J.M. (1988) Curr. Top. Cell. Regul. 29, 129–216.
[8] Braun, U., Habermann, B., Just, I., Aktories, K. and Vandekerckhove, K. (1989) FEBS Lett. 243, 70–76.
[9] Narumiya, S., Sekine, A. and Fujiwara, M. (1988) J. Biol. Chem. 263, 17255–17257.
[10] Kikuchi, A., Yamamoto, K., Fujita, T. and Takai, Y. (1988) J. Biol. Chem. 263, 16303–16308.
[11] Chardin, P., Boquet, P., Madaule, P., Popoff, M.R., Rubin, E.J. and Gill, D.M. (1989) EMBO J. 8, 1087–1092.
[12] Aktories, K., Weller, U. and Chhatwal, G.S. (1987) FEBS Lett. 212, 109–113.
[13] Aktories, K. and Frevert, J. (1987) Biochem. J. 247, 363–368.
[14] Aktories, K., Roesener, S., Blaschke, U. and Chhatwal, G.S. (1988) Eur. J. Biochem. 172, 445–450.
[15] Rubin, E.J., Gill, D.M., Boquet, P. and Popoff, M.R. (1988) Mol. Cell. Biol. 8, 418–426.
[16] Birchmeier, C., Brock, D. and Wigler, M. (1985) Cell 43, 615–621.
[17] Korn, L.J., Siebel, C.W., McCormick, F. and Roth, R.A. (1987) Science 236, 840–843.
[18] Paterson, H.F., Self, A.J., Garrett, M.D., Just, I., Aktories, K. and Hall, A. (1990) J. Cell Biol. 111, 1001–1007.
[19] Garret, M.D., Self, A.J., van Oers, C. and Hall, A. (1989) J. Biol. Chem. 264, 10–13.
[20] Richardson, C. and Potter, R.L. (1983) Dev. Biol. 99, 240–247.
[21] Kita, T., Brown, M.S. and Goldstein, J.L. (1980) J. Clin. Invest. 66, 1094–1107.
[22] Laemmli, U.K. (1970) Nature 227, 680–685.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Hancock, J.F., Magee, Childs, J.E. and Marshall, C.J. (1989) Cell 57, 1167–1177.

[25] Chardin, P., Madaule, P. and Tavitian, A. (1988) Nucleic Acids Res. 16, 2717.
[26] Yeramian, P., Chardin, P., Madaule, P. and Tavitian, A. (1987) Nucleic Acids Res. 15, 1869.
[27] Sekine, A., Fujiwara, M. and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605.
[28] Pai, E.F., Kabsch, W., Krengel, U., Holmes, K.C., John, J. and Wittinghofer, A. (1989) Nature 341, 209–214.
[29] Maller, J.L. and Krebs, E.G. (1977) J. Biol. Chem. 252, 1712–1718.
[30] Aktories, K., Just, I., Müller, H., Wiegers, W. and Traub, P. (1990) Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl. 341, R33.
[31] Hausen, P., Wang, Y.H., Dreyer, C. and Stick, R. (1985) J. Embryol. Exp. Morphol. Suppl. 89, 17–34.