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Receptor mediated mineralocorticoid action in alga cell mutants

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The multiplication of *Chlamydomonas* cells can be arrested by the spirolactone derivative RU 26752 and this is fully reversible by the natural hormone aldosterone. Continuous growth in the presence of RU 26752 led to the isolation of a population subsequently resistant to the action of mineralocortoid analogues, due possibly to the selection of mutant cells. Immunophotochemical evidence is provided for a 52 kDa protein that possesses functional steroid and DNA binding domains. Alga cells therefore appear to respond to steroid hormones in a manner similar to the mammalian systems, possibly via a receptor that may represent a pygmy ancestor of the latter day steroid receptor superfamily.

Alga; *Chlamydomonas*; Mineralocorticoid; Receptor; Aldosterone; Spirolactone; RU 26752

1. INTRODUCTION

Steroid hormones bind to cellular receptors that act as *trans*-activating signals for selective genetic modulation specific to the appropriate cell type [1–3]. Such receptors are members of a superfamily of proteins with some 30 known candidates at various evolutionary levels in the animal world [1–3].

The mineralocorticoid hormone receptor (MCR) is the largest member of this superfamily with 984 residues in the gene cloned from rat brain [4]. Its existence was initially demonstrated by radioligand binding and physiological assays in the epithelial cells of several amphibian [5,6] and mammalian [7–9] organs but its possible presence in the plant kingdom remains unknown.

The synthesis of a ligand specific to MCR [10,11] permitted biochemical purification of the receptor from mammalian organs [12,13] which was then used for the genesis of a polyclonal antiserum directed against the MCR that could also be photolabelled for the very first time [14,15]. This permitted us to screen a number of cell types for possible MCR-like carriers.

Chlamydomonas is a unicellular fresh water alga with many unique features at the cross roads of evolutionary scale such as plant-like chloroplasts, protozoan flagella, and rhodopsin characteristic of the mammalian eye [16]. We therefore investigated mineralocorticoid hormone action in this context.

We show here that *Chlamydomonas* cells are physiologically responsive to mineralocorticoids, possibly via a 52 kDa carrier, and are endowed with a mechanism to overcome the adverse effects of animal signals. The evolutionary roots of receptor-mediated steroid hormone action therefore appear to be rather ancient.

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2. MATERIALS AND METHODS

Chlamydomonas reinhardtii (wild type) cells were grown in Tris-acetate-phosphate medium at 27°C with alternate 12 h light and dark cycles [16]. Growth was assessed in both solid and liquid medium in the presence of various concentrations of steroids of choice for a total of 48 h. Cell density was quantitated by absorbance at 660 nm, and by macrophotography. A mutant population was isolated after 10 days of growth in the presence of 10 μM RU 26752.

Cytosol was prepared by cell extraction in 10 mM phosphate buffer pH 7.4 containing 0.01 mM PMSF, 20 mM albumin and 10 mM DTT, using a French press. Total protein was precipitated with 50% ammonium sulphate and redissolved in the same buffer. Samples of 60 μg were electrophoresed on 15% polyacrylamide gels calibrated with markers of known molecular weight and stained with Coomassie brilliant blue [14–16].

For Western blots, proteins separated by SDS-PAGE were electrotransferred to nitrocellulose membranes (Millipore), blocked for 1 h with non-fat dry milk at 37°C, and finally flooded with a rat renal anti-MCR antibody raised in the rabbit for 2 h at 4°C [14,15]. Blots were saturated with sheep anti-rabbit biotinylated antibody for 90 min, followed by 90 min in the presence of streptavidin-biotinylated horseradish peroxidase complex, and finally developed with 4-chloro-1-naphthol-hydrogen peroxide-methanol, as described in detail recently [14,15].

For photoaffinity, alga cytosol was incubated with 50 nM [^{3}H]-R-5020 (promegestone) alone or in presence of 5 μM RU 26752, and then irradiated at < 1 cm for 3 min at 4°C with a double bore mercury vapour lamp (Jelight). The samples were electrophoresed along with radiolabelled but non-irradiated cytosol, and free radioligand, as controls. The gels were fixed with water/acetic acid, flooded with Amplify (Amersham), dried, exposed to Hyperfilm MP in a fluorographic holder for two weeks at −80°C, and finally developed with a Kodak processor [14,15].

DNA-cellulose assays were performed on cytosol equilibrated with 20 nM [^{3}H]-RU 26752 for 2 h at 2–4°C, and charcoal-treated to eliminate free steroids. One series was left in melting ice whereas the other was allowed to stand at the room temperature (23°C) for 45 min. Aliquots of 500 μl were mixed with 200 μl of DNA-cellulose suspension (Sigma; 7 mg/g) and incubated for 45 min at 4°C. After three

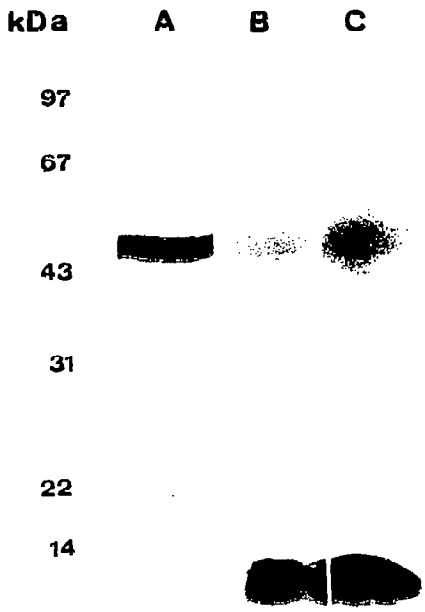

Fig. 1. Immunophotochemical evidence for a mineralocorticoid hormone receptor-like binder in *Chlamydomonas*. Markers of known molecular weight were electrophoresed with 60 μg alga cytosol for Western blot analysis (A) and photochemistry with either [^{3}H]-R 5020 alone (C) or in the presence of an excess of cold RU 26752 (B).

washes with 50 mM Tris-HCl pH 8, containing 12 mM MTG, 10 mM KCl, 20% glycerol, the pellets were extracted twice with this Tris buffer plus 1 M KCl. The combined extracts (1 ml) were counted in 10 ml Picofluor [10,11]. The results represent the average of three separate determinations.

Radioinert and [^{3}H]-RU 26752 (50 Ci/mM, reference X 4025 A) were kindly provided by Dr. D. Philibert, Roussel-Uclaf, Romianville. Aldosterone was purchased from Sigma. [^{3}H]-R 5020 (84.7 Ci/mM; lot 2668-062) and cold promegestone were obtained from New England Nuclear Corporation.

3. RESULTS AND DISCUSSION

Mammalian receptors for steroid hormones vary in length but consist of a well defined domain structure [1–3]. Data in Fig. 1 show a single band of about 52 kDa when *Chlamydomonas* cytosol was analyzed by the Western blot technique with a polyclonal anti-MCR serum raised in the rabbit against rat renal antigen which, under these conditions, was resolved as a 98 kDa band [14,15] in close agreement with the theoretical molecular size of 107 kDa [4].

A steroid binds to the C-terminal, hormone-binding domain (HBD) of the receptor via noncovalent forces [1–3] but it can be photochemically cross-linked to the HBD as a test of the receptor identity [14,15]. It is clear from Fig. 1 that [^{3}H]-R 5020 could be photochemically linked to a 52 kDa carrier in *C. reinhardtii* cytosol. The labelling was greatly diminished if cold RU 26752, specific to MCR, was allowed to compete with radioactive R 5020 for receptor occupancy, similar to the situation in mammalian organs where a 98 kDa MCR was recently photolabelled [14]. Radioligand alone, or non-irradiated cytosol–[^{3}H]promegestone mixtures did not label either the mammalian receptor [14,15], or the alga carrier (not shown), confirming technical specificity. Thus, a functional HBD is obviously present on the 52 kDa alga carrier.

The DNA-binding domain (DBD) is highly conserved throughout the steroid hormone receptor superfamily and has been assessed most frequently by the quantity of radioactivity bound to DNA-cellulose following receptor transformation by heat, salt, or ATP [10,11]. The affinity of the alga complex for DNA-cellulose increased by 50% after 45 min at 23°C compared to controls at 2–4°C maintained in an ice bath (626 vs. 329 cpm, respectively), despite the possible occupancy of the *Chlamydomonas* carrier by endogenous ligands expected to interfere with this assay.

The presence of functional HBD and DBD on the 52 kDa alga carrier suggested that steroids should influence cell function. Data in Fig. 2 show that RU 26752 blocked the growth of *Chlamydomonas reinhardtii* in a dose-dependent manner during the first 24 h, followed by some recovery by 48 h. This could mean that RU 26752-mediated inhibition of cell growth was reversible due to a metabolic adaptation. Alternatively, it is conceivable that the resistant cell is either receptor deficient or contains a mutant receptor protein. The natural mammalian hormone aldosterone did not inhibit cell growth under these conditions but reversed the inhibitory effect of RU 26752. This indicates the presence of endogenous ligands that were required for the alga cell survival and that could not be displaced from the 52 kDa carrier by the mineralocorticoid or were merely replaced by aldosterone.

Data in Fig. 3 show that a population of cells surviving the highest dose of spirolactone (Fig. 2) was subsequently refractory to RU 26752 action for 10 days (five times the growth cycle of wild-type cells) on a solid medium (Fig. 3). Microscopic examination did not reveal any difference in cell morphology under these conditions so the action of steroids appears to be primarily cytostatic. Both the wild-type and the resistant strains exhibited qualitatively similar MCR-specific staining in Western blots (Fig. 3), suggesting a mutant receptor in the latter.

It follows from the foregoing that techniques of mammalian endocrinology are fully applicable to analyze plant functions. In fact, alga cell growth in vitro may even form an assay for screening the physiological activity of mineralocorticoids, and possibly of other steroids. This confirms and extends receptor-mediated steroid action in a typical plant cell context [15].

Despite the apparent similarity of physiological action, the alga carrier is only half the size of the mammalian receptor. It appears to accept a number of ligands, including those required for normal growth of the alga. Conceivably, a pygmy ancestor of great versatility in the

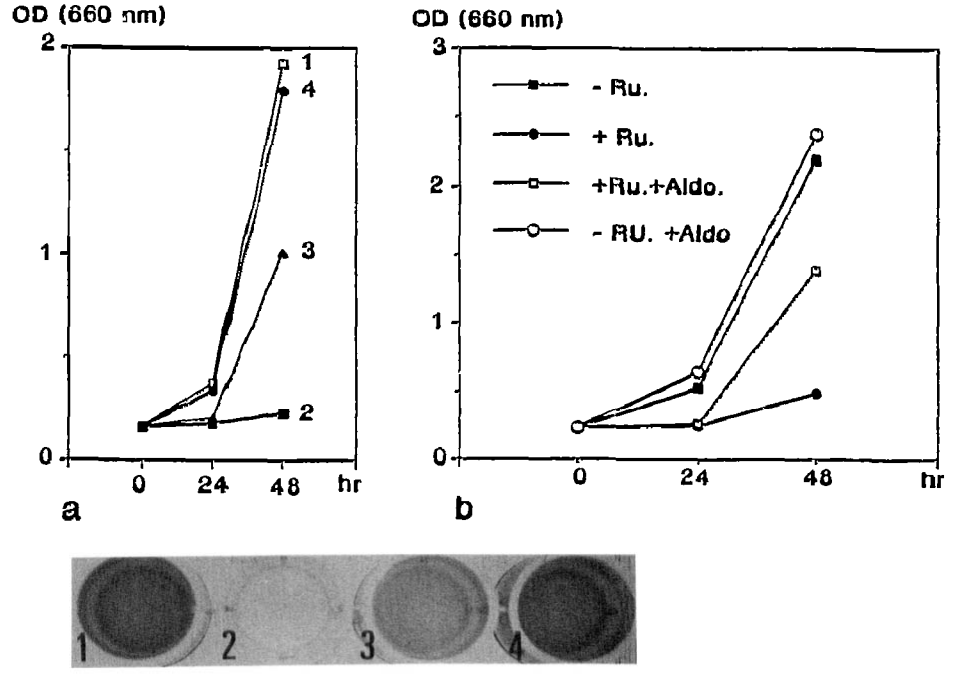

Fig. 2. Mineralocorticoids influence *Chlamydomonas* cell growth. *C. reinhardtii* cells were cultivated in presence of 1% ethanol (1), 10 μM (2), 2 μM (3), or 400 nM (4) RU 26752 alone (panel a) for a total of 48 h; 10 μM of aldosterone and/or RU 26752 were used for panel b. Photographs of the growth pattern in panel A are also shown.

plant cell could later have specialized into a family of larger carriers to accommodate mammalian complexity and diversity, whereas the mutant receptor would assure the survival of the alga under adverse vicissitudes.

Interestingly, *Chlamydomonas* cells respond to to light [17] and contain the 'S' antigen involved in phototransduction [16]. This further confirms that mammalian counterparts appear to be conserved down to the simple unicellular alga with much economy to mechanisms regulating genetic diversity. If this be the case, the cloning of the versatile, receptor-like protein reported here, may reveal new patterns of macromolecular structure and function, such as the glucocorticoid inducible gene expression system in chimaeric constructs [18]. Indeed, steroids are produced by a number of plant species [19] and fungal pathogenicity has even been correlated with a putative binder. Such considerations have important implications regarding the evolution and the ancestry of the steroid receptor superfamily.

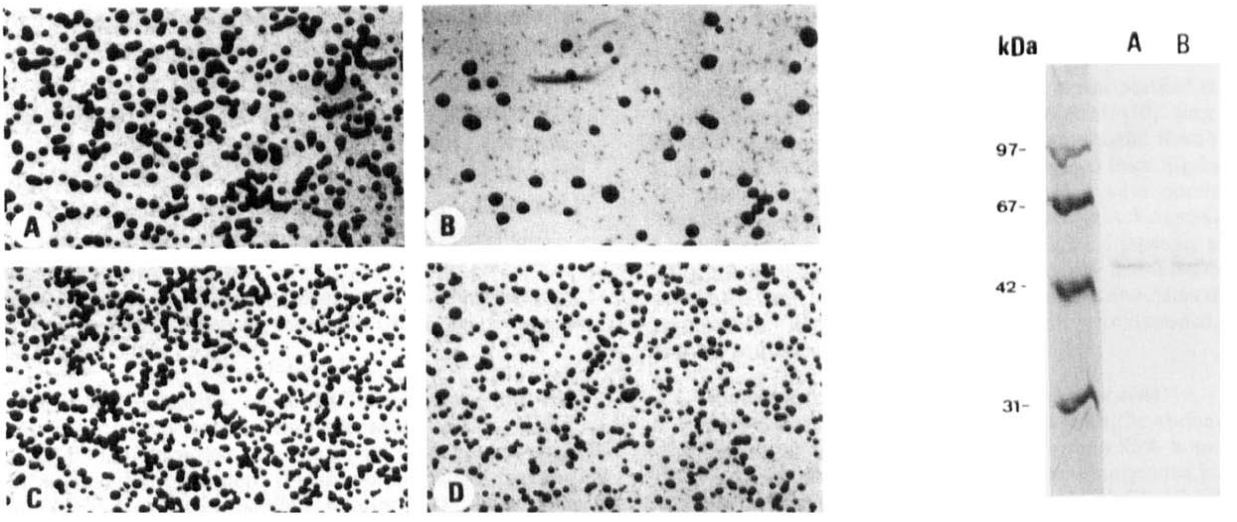

Fig. 3. Alga cells refractory to the spirolactone possess a mutant carrier. Cells were grown for 10 days on 10% Agarose-phosphate buffer in presence of ethanol alone (A) or 10 μM RU 26752 (B). Colonies surviving the spirolactone in B were then cultured in control medium (C) or that containing 10 μM RU 26752 (D) for 10 days. Cytosol from the sensitive (A) and resistant (B) strains was analyzed by immunoblots as in Fig. 1.

REFERENCES

[1] Seagraves, W.A. (1991) Cell 67, 225–228.
[2] Wahli, W. and Martinez, E. (1991) FASEB J. 5, 2243–2249.
[3] Agarwal, M.K. (1990) Die Natur 77, 170–177.
[4] Patel, P.D., Sherman, T.G., Goldman, D.J. and Watson, S.J. (1989) Mol. Endocrinol. 3, 1877–1885.
[5] Sharp, G.W.G. and Leaf, A. (1966) Physiol. Rev. 46, 593–633.
[6] Alberti, K.G.M.M. and Sharp, G.W.G. (1969) Biochim. Biophys. Acta 192, 335–346.
[7] Snart, R.S. and Taylor, E. (1978) J. Physiol. 274, 447–454.
[8] Agarwal, M.K. (1975) Nature 254, 623–625.
[9] Nichols, N.R., Obert, D.F. and Meyer, W.J. (1985) Life Sci. 36, 1653–1660.
[10] Agarwal, M.K. and Kalimi, M. (1988) Biochim. Biophys. Acta 964, 105–112.
[11] Agarwal, M.K. and Kalimi, M. (1988) Biochem. Biophys. Res. Commun. 150, 449–455.
[12] Lazar, G., Pagano, M. and Agarwal, M.K. (1990) Intnl. J. Biochem. 22, 621–630.
[13] Lazar, G., Pagano, M. and Agarwal, M.K. (1990) Biochim. Biophys. Acta 1033, 41–48.
[14] Mirshahi, M., Pagano, M., Mirshahi, A. and Agarwal, M.K. (1992) Biochim. Biophys. Acta 1120, 17–23.
[15] Mirshahi, M., Mirshahi, A., Nato, A. and Agarwal, M.K. (1992) Biochem. Biophys. Res. Commun. 186, 1102–1107.
[16] Mirshahi, M., Nato, A., Razaghi, A., Mirshahil, A. and Faure, J.P. (1991) Compte Rendu Acad. Sci. (Paris) 312, 441–448.
[17] Harz, H. and Hegemann, P. (1991) Nature 351, 489–491.
[18] Schena, M., Lloyd, A.M. and Davis, R.W. (1991) Proc. Nat. Acad. Sci. USA 88, 10421–10425.
[19] Lenard, J. (1992) Trends Biochem. Sci. 17, 147–150.