Journal of Cellular Biochemistry 61:167–171 (1996)

ARTICLES

Osmolarity Is an Independent Trigger of *Acanthamoeba castellanii* Differentiation

John S. Cordingley, Rebecca A. Wills, and Clarence L. Villemez

Department of Molecular Biology, University of Wyoming, Laramie, Wyoming 82071-3944

Abstract Like many yeasts, bacteria, and other sporulating microorganisms, *Acanthamoeba castellanii (Neff)*, a free-living amoeba with pathogenic relatives, differentiates into a dormant form when deprived of nutrients. *Acanthamoeba* cysts redifferentiate into trophozoites when food is resupplied. We report here that *Acanthamoeba* encystment is also triggered by elevated osmolarity, and that osmolarity and cell surface receptor binding are synergistic in triggering differentiation. Additions of sodium chloride or glucose to rich growth media were used to produce specific osmolarity increases and similar encystment results were obtained with either additive. Although many organisms, including *Acanthamoeba* and mammalian cells, have been shown to adapt to hyperosmolar conditions, this is the first demonstration that hyperosmolarity can be a primary differentiation signal. © 1996 Wiley-Liss, Inc.

Key words: encystment, differentiation, amoeba, osmolarity

Acanthamoeba castellanii (Neff) is a soil amoeba which can differentiate from a free-living single-celled predator (trophozoite) into a metabolically inactive, walled form (cyst) [see reviews by Byers, 1979; Byers et al., 1991; Weisman, 1976]. It has close relatives which are human pathogens. The bulk of research using this organism has been motivated by these two characteristics.

Acanthamoeba encystment is functionally reminiscent of *Bacillus subtilis* [Errington, 1993], *Saccharomyces cerevisiae* [Mitchell, 1994], and *Dictyostelium discoideum* [Francis et al., 1977] sporulation. In those organisms, partial (e.g., nitrogen starvation) or complete starvation is considered to be the environmental condition responsible for inducing sporulation. *Acanthamoeba* encystment is also clearly triggered by starvation. In the laboratory, axenically grown *Acanthamoebae* can be quantitatively converted to cysts by transfer to an aerated, nutrient-free, buffered salt solution that is roughly isoosmolar with growth media [Neff et al., 1964]. But, unlike the other organisms mentioned above, there is some confusion about the environmental signals that induce *Acanthamoeba* to differentiate. For example, this statement was made in a recent review on *Acanthamoeba* [Bottone, 1993]: "The factors that control encystment and excystment are not known. Generally, adverse environmental conditions induce the trophozoites to encyst. . . ."

Increased osmolarity has been shown to cause cellular adaptive changes in many organisms, for example bacteria [Csonka, 1989], yeast [Maeda et al., 1994], and mammalian cells [Han et al., 1994; Galcheva-Gargova et al., 1994]. *A. castellanii (Neff)* also adapts to hyperosmolar conditions [Geoffrion and Larochelle, 1984], partially by increasing the intracellular concentration of amino acids. Evidence derived from experimentation with bacteria, yeast, and mammalian cells indicates that protein kinase signal systems are involved in producing the hyperosmolarity cellular changes, and these mechanisms appear to be similar to those involved in other types of "stress" adaptation [Mager and Varela, 1993]. Hyperosmolarity has been shown to affect the cellular end product of *Polysphondylium pallidum* starvation-induced differentiation [Toama and Raper, 1967]. Band [1963] reported that hyperosmolarity was a factor, along with nutrient deprivation, in *Hartmanella rhysodes* encystment. However, the induction of differentiation by increased osmolarity

Received September 4, 1995; accepted September 7, 1995.

John S. Cordingley's present address is Merck Research Laboratories, WP44-I130, West Point, PA 19486.

Address reprint requests to C.L. Villemez, Department of Molecular Biology, University of Wyoming, Laramie, WY 82071-3944.

alone has not, to our knowledge, been reported previously for any organism.

MATERIALS AND METHODS

A. castellanii (Neff) was obtained from the American Type Culture Collection (Rockville, MD) and grown in axenic medium as described [Neff et al., 1964; Villemez et al., 1985]. The final concentration of glucose in the medium was 3% (w/v) except for the experiment described in Figure 2 where the final concentration of glucose was 1.5%. Monoclonal antibody A9 was purified from ascites fluid as described previously [Yang and Villemez, 1994]. The antibody was pure as indicated by SDS gel electrophoresis. Additions and manipulations are described in the text. Trophozoites were counted in phosphate buffered saline using a Coulter counter or visually with a hemocytometer. Cysts were counted in 0.9% NaCl, 0.5% SDS. Under these conditions no trophozoites are detectable with either counting method. All experiments were performed more than once. All results obtained were similar to those reported here.

Care in preparing growth media was required to obtain consistent doubling times, the lack of growth lag when inoculating amoebae into fresh media, and consistent results in the osmolarity experiments. The main factor in this careful preparation related to osmolarity occurred during sterilization of the growth media. Significant and variable amounts of water were lost during autoclaving of media thereby altering the osmolarity of growth media. To correct this we weighed the media and container before and after autoclaving and added back sterile water to compensate for that loss.

RESULTS

Sodium chloride (5M) was added to cultures of *A. castellanii* growing logarithmically in axenic medium containing 3% glucose to give a final salt concentration of 125 mM. An equal volume of water was added to similar cultures as control. The amoebae cease dividing within 24 h of when the salt is added (Fig. 1A), regardless of cell concentration at the addition time, and the cell number never doubles. Cysts begin to appear in the cultures shortly after the addition of salt (Fig. 1B), and encystment continues in these cultures until 50–80% of the cells have encysted. The control cultures continue growing logarithmically until they reach a density of 1.5×10^7 cells/ml, with cysts being essentially undetectable in these cultures. Mature, viable cysts do not form in the control cultures even after several weeks of incubation. Several days after stationary phase the cells begin to lyse, the cultures becoming full of cell debris. Cell lysis continues in the stationary phase cultures until all of the remaining cells have the microscopic appearance of immature cysts, none of which are viable.

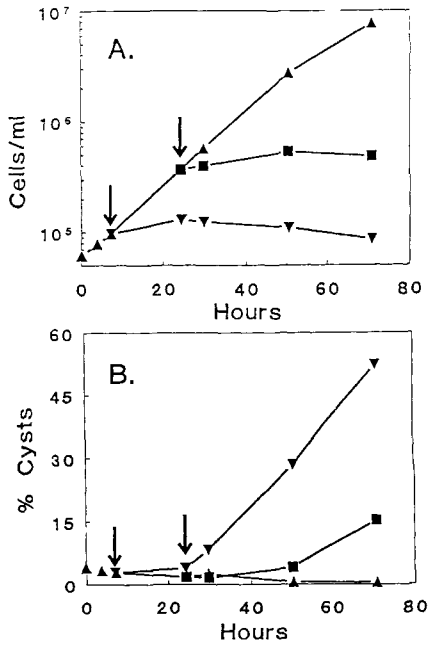

Fig. 1. Adding salt to growing Acanthamoebae induces encystment. **A:** Cells/ml in cultures at the times indicated. Vertical arrows indicate salt addition (125 mM NaCl final concentration). No added salt (▲), 125 mM NaCl added to 10^5 cells/ml (▼), 125 mM NaCl added to 3.7×10^5 cells/ml (■). **B:** Percentage SDS-resistant cysts at the indicated times. Symbols same as in A.

The cysts formed in response to added salt are microscopically indistinguishable from cysts formed in conventional encystment medium. These cysts were washed with 1% NP-40 to lyse any remaining trophozoites and placed into fresh medium where they excysted and grew normally after 5–7 days.

To distinguish between some random effect of NaCl, as opposed to increasing the osmolarity of medium, the same experiments were performed with the addition of glucose substituting for NaCl. For these experiments, 60% glucose solutions were added to cultures with a cell density of 10^5/ml to give final glucose concentrations of 7.5%. The cultures had been growing in media containing 3% glucose. The additional 4.5% glucose produces the same osmolarity increase (0.25

TABLE I. Subthreshold Osmolarity Increases Depress Growth Rate*

mOs increase	Relative growth rate (%)	Cyst proportion (%)
0	100	1
100	76	2
150	57	2
200	39	10
250	0	44
300	0	85
350	0	94

*Culture media, cell and cyst number determination was that described in Materials and Methods. Sufficient NaCl (5M) was added to produce the osmolarity increases indicated. Relative growth rate was the ratio of the slopes of semi-log plots of cell number vs. time for 37.5 h following the additions. During the 37.5 h of the experiment the control culture increased in cell number from 1.24×10^5 to 1.72×10^6. The proportion of cysts was determined 37.5 h following the additions.

osmolar) as 125 mM NaCl. These cultures, in which the osmolarity increase was produced by glucose, encysted in a manner similar to that shown in Figure 1.

A 0.25 osmolar increase appears to be a threshold of sorts. That is the approximate osmolarity increase which causes cell division to stop completely, and a very large proportion of cells eventually encyst. Osmolarity increases larger than 0.25 cause cell division to stop more rapidly and encystment occurs at a faster rate (Table I). Osmolarity increases below 0.2 do not cause cell division to stop, albeit the growth rate is slowed, and insignificant encystment occurs. With an osmolarity increase of 0.2 growth never ceases completely but a small but significant amount of cells encyst. Similar results are obtained when glucose is used to raise the osmolarity of the media.

To determine whether interactions between increased osmolarity and other conditions known to induce encystment could be found, experiments were performed using various decreases in growth media glucose in combination with increased osmolarity as well as experiments using additions of various concentrations of monoclonal antibody known to induce encystment in combination with increased osmolarity. We could find no indications of synergy between food withdrawal and increased osmolarity (data not shown), but increased osmolarity and monoclonal antibody addition were synergistic stimuli of encystment (Fig. 2). Neither 0.05M NaCl (an increase of 100 mOs) or the presence of 5 μg/ml

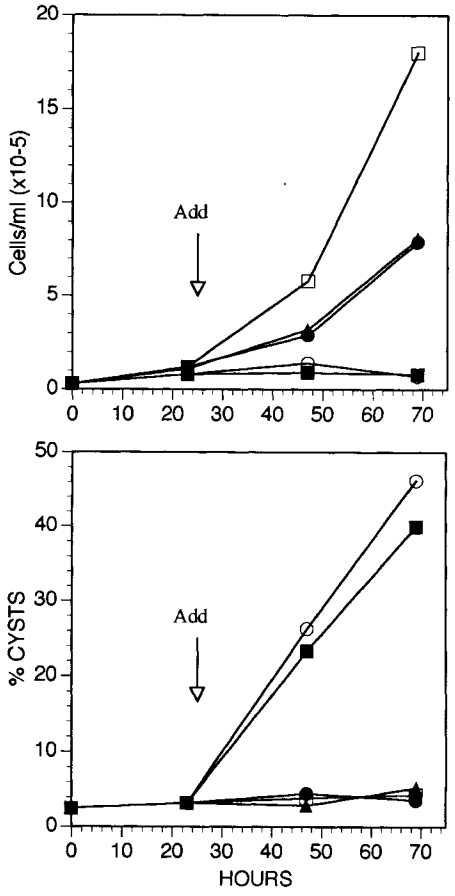

Fig. 2. Synergy between increased osmolarity and cell surface receptor binding in the induction of encystment. Trophozoites in midlog phase were collected, washed with fresh growth media, resuspended in fresh growth media, identical aliquots distributed into individual flasks, and incubated at 30°C. At the time indicated by the arrow additions were made to produce the following final concentrations: 0.125M NaCl (■), 0.05M NaCl (●), 5 μg/ml monoclonal antibody A9 (▲), 0.05M NaCl + 5 μg/ml A9 (○), or no additions (☒). NaCl was added as a 5M aqueous solution, and A9 as a 1 mg/ml solution in phosphate buffered saline. Cell number and cyst number were measured as described in the text.

monoclonal antibody A9 triggered encystment, although both slowed growth rate somewhat in accord with previous observations. However, when present in combination encystment was triggered. The rate of encystment with the combination was comparable to that induced by a 250 mOs increase (0.125M NaCl).

DISCUSSION

These results demonstrate that osmolarity increases of 0.25 osmolar are sufficient to cause the great majority of *A. castellanii* (*Neff*) cells to commence differentiation and produce normal viable cysts in rich growth medium. The osmo-

larity increases were produced, with similar results, by additions of NaCl or glucose. The chemistries of those two substances and their effect on living cells are quite disparate. One of them, glucose, is a required nutrient for this organism under axenic growth conditions. Considering the quantitative correlation in conjunction with the chemical disparity it is clear that the only common factor in the addition of these two substances to growth media is the increase in osmolarity. Further, it has been demonstrated that *A. castellanii* (*Neff*), as with other cell types, is capable of adapting to hyperosmolar conditions [Geoffrion and Larochelle, 1984], i.e., encystment is not a necessary survival response for these cells any more than it is for yeast. Consequently, there is little doubt that the amoebae are perceiving osmolarity change as a primary stimulant for differentiation. The corresponding environmental condition is probably a reduction in water due to evaporation.

Osmolarity increases greater than 0.25 result in a somewhat higher final proportion of mature cysts but, more impressively, a much faster rate of cyst formation. Osmolarity increases below 0.2 do not trigger significant numbers of cells to encyst, but cell division rate is lowered. These circumstances suggest that the cellular commitment to differentiate includes indecisive stages, i.e., it is not a simple on-off switch. Supporting this suggestion are results from the binding of a cell surface receptor that triggers encystment in *Acanthamoeba* [Villemez et al., 1985; Yang and Villemez, 1994]. Continuous presence of the ligand caused essentially all the cells to differentiate while a single binding event stopped cell division for 18 h, inducing no encystment and following which time the cells reentered log phase.

The confusion about *Acanthamoeba* encystment appears to be partially caused by numerous reports, most relatively old, studying the effect of a large variety of additives and conditions on starvation-induced differentiation. Once differentiation is triggered by starvation, one would predict that anything affecting cellular biochemistry might influence the rate and/or success ratio of the process. Consequently, no experimentation conducted in that fashion can reliably be interpreted in terms of signals for *Acanthamoeba* encystment. Further adding to the confusion, various macromolecule synthesis inhibitors, mainly DNA synthesis inhibitors, have been reported to induce *Acanthamoeba* encystment in full nutrient media [for a review see Byers et al., 1991]. The latter studies have not been brought to an experimental conclusion, primarily because the reported effects are not completely reproducible (perhaps due to mutation or adaptation of the organism). Regardless, since alterations of DNA transcription must take place during differentiation, it is impossible to interpret these results in terms of signals for encystment under any circumstances. Also, a ready explanation for the observation is available by analogy with another system: the induction of *Bacillus subtilis* spore formation by decoyinine [Mitani et al., 1977]. Decoyinine is an inhibitor of GMP synthetase, and causes a drop in the intracellular concentrations of GDP and GTP. The bacteria presumably uses guanine nucleotide levels as correlate of nutritional status [Freese, 1981], and a guanine nucleotide-binding protein is probably the initial receptor in the signal cascade that leads to sporulation. That is, the bacteria does not sporulate in response to "antibiotics" but to that particular antibiotic because it causes an intracellular effect that mimics starvation. The same situation seems likely to apply to *Acanthamoeba's* reported responses to macromolecule synthesis inhibitors.

Previously, we reported that *A. castellanii* is stimulated to encyst by the binding of a cell surface receptor, and excystment is inhibited by binding of the same, or a very similar, receptor called ESP (Encystment Stimulating Protein) [Villemez et al., 1985; Yang and Villemez, 1994]. The natural ligand for ESP is unknown. Since ESP's characteristics appear to exclude it from being involved in the starvation response, and an osmolarity detector is apparently required to account for the results presented here, ESP is a logical candidate for the *Acanthamoeba* osmolarity receptor. The fact that increased osmolarity and ESP binding is apparently synergistic in triggering differentiation adds some support to the candidacy of ESP as an osmolarity receptor.

While many conditions can influence *Acanthamoeba* encystment once it has been initiated, results prior to this work provided starvation as the only demonstrated environmental stimulus. The work reported here adds an additional independent stimulus capable of triggering *Acanthamoeba* encystment: increased osmolarity.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health grant CA52562.

REFERENCES

Band RN (1963): Extrinsic requirements for encystation by the soil amoeba, *Hartmannella rhysodes*. J Protozool 10: 101–106.

Bottone EJ (1993): Free-living amebas of the genera *Acanthamoeba* and *Naegleria:* An overview and basic microbiologic correlates. Mount Sinai J Med 60:260–269.

Byers RJ (1979): Growth, reproduction, and differentiation in *Acanthamoeba*. Int Rev Cytol 61:283–337.

Byers TJ, Kim BG, King LE, Hugo ER (1991): Molecular aspects of the cell cycle and encystment of *Acanthamoeba*. Rev Infect Dis 13(Suppl. 5):S378–S384.

Csonka LN (1989): Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53:121–147.

Errington J (1993): *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. Microbiol Rev 57:1–33.

Francis DW, Eisenberg RM, MacInnes M (1977): Genetics and development in *Dictyostelium* and *Polysphondylium*. In O'Day DH, Horgen PA (eds): "Eucaryotic Microbes as Model Developmental Systems." New York: Marcel Dekker, Inc., pp 155–177.

Freese E (1981): Initiation of bacterial sporulation. In Levinson HS, Sonenshein AL, Tipper DJ (eds): "Sporulation and Germination." Washington, DC: Am Soc Microbiol, pp 1–12.

Galcheva-Bargova Z, Derijard B, Wu I-H, Davis RJ (1994): An osmosensing signal transduction pathway in mammalian cells. Science 265:806–808.

Geoffrion Y, Larochelle J (1984): The free amino acid contribution to osmotic regulation in *Acanthamoeba castellanii*. Can J Zool 62:1954–1959.

Han J, Lee J-D, Bibbs L, Ulevitch RJ (1994): A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808–811.

Maeda T, Wurgler-Murphy SM, Saito H (1994): A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369:242–245.

Mager WH, Varela JCS (1993): Osmostress response of the yeast *Saccharomyces*. Mol Microbiol 10:253–258.

Mitani T, Heinze JE, Freese E (1977): Induction of sporulation in *Bacillus subtilis* by decoyinine or hadacidin. Biochem Biophys Res Commun 77:1118–1125.

Mitchell AP (1994): Control of meiotic gene expression in *Saccharomyces cerevisiae*. Microbiol Rev 58:56–70.

Neff RJ, Ray SA, Benton WF, Wilborn M (1964): Induction of synchronous encystment (differentiation) in *Acanthamoeba sp*. Methods Cell Physiol 1:55–83.

Toama MA, Raper KB (1967): Microcysts of the cellular slime mold *Polysphondylium pallidum*. I. Factors influencing microcyst formation. J Bact 94:1143–1149.

Villemez CL, Carlo PL, Russell MA (1985): Differentiation in *Acanthamoeba castellanii* is induced by specific monoclonal antibodies. J Cell Biochem 29:373–379.

Weisman RA (1976): Differentiation in *Acanthamoeba castellanii*. Ann Rev Microbiol 30:189–219.

Yang S, Villemez CL (1994): Cell surface control of differentiation in *Acanthamoeba*. J Cell Biochem 56:592–596.