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Original Article

Expression of Bacteriorhodopsin in Sf9 and COS-1 Cells

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We report studies on the expression of the archaebacterial membrane protein bacteriorhodopsin in Sf9 insect cells and in COS-1 mammalian cells. In both cell systems, the apoprotein bacterio-opsin was expressed at levels of ~ 1 $\mu g/10^6$ cells. Immunofluorescence studies showed that the expressed protein was accumulated in the endoplasmic reticulum. However, upon addition of *all-trans* retinal to membranes isolated from either Sf9 or COS-1 cells expressing bacterio-opsin, the characteristic bacteriorhodopsin chromophore (λ_{max} at ~560 nm) was rapidly generated. This is in contrast to bacterio-opsin expressed in *E. coli*, which cannot be functionally reconstituted with retinal unless it is first denatured, and then renatured in vitro. These studies demonstrate that the bacterio-opsin expressed is correctly folded and show that localization of a heterologously expressed membrane protein in the endoplasmic reticulum does not necessarily imply that it is misfolded.

KEY WORDS: Membrane protein; rhodopsin; folding; intracellular transport.

INTRODUCTION

Membrane proteins from one organism can often be successfully expressed in cells from other organisms. In many cases, the heterologously expressed proteins are functional, and on occasion, they can be overexpressed at levels significantly above the amounts expressed in the native organism (Grisshamer and Tate, 1995). However, the outcome of such experiments is somewhat unpredictable. Frequently, the heterologously expressed protein is misfolded and nonfunctional. In eukaryotic cell expression systems, misfolded membrane proteins generally do not exit the endoplasmic reticulum (ER) (Gething and Sambrook, 1990). Because of the large number of studies which have shown that exit of membrane and secreted proteins from the ER requires correct folding, it is generally believed that ER localization implies that the heterologously expressed protein is misfolded.

The archaebacterial membrane protein bacteriorhodopsin provides a good model system to further investigate the correlation between misfolding and ER localization. Bacteriorhodopsin, a light-driven proton pump which is found in the purple membrane of the extreme halophile *H. salinarium*, is a very well-studied membrane protein (for reviews see *J. Bioenerg. Biomembr.* 24, 1992). The apoprotein has been successfully expressed in *E. coli* (Karnik *et al.*, 1987), in the yeast *S. cerevesiae* (Lang-Hinrichs *et al.*, 1994) and *S. pombe* (Hildebrand *et al.*, 1989), in *Xenopus* oocytes (Nagel *et al.*, 1995), and in cell-free, *in vitro* translation systems (Sonar *et al.*, 1993). However, with the exception of the proteins expressed in *S. pombe* and in oocytes, the expressed protein appears to be misfolded, and a functional bacteriorhodopsin chromophore cannot be generated by the addition of *all-trans* retinal to the cell membranes. Thus, to obtain correctly folded bacterio-opsin from proteins expressed in *E. coli*, it is necessary to first denature the protein, and then renature it in the presence of an appropriate mixture of lipids and detergents (Braiman *et al.*, 1987).

Here, we report studies on the expression of bacterio-opsin in Sf9 cells and in COS-1 cells, systems which are commonly used for the heterologous expression of many membrane proteins. In contrast to the plasma membrane localization observed in *S. pombe* cells and in frog oocytes, bacterio-opsin expressed in

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Sf9 cells and in COS-1 cells is predominantly localized to the ER. However, the expressed protein binds retinal to generate a native-like bacteriorhodopsin chromophore.

MATERIALS AND METHODS

Antibodies

The monoclonal antibody bR 114 (Kimura *et al.*, 1982) directed against the C-terminus of bacteriorhodopsin was used to probe for bacterio-opsin expression. Mouse monoclonal IgG antibody raised against β-galactosidase was purchased from Promega (Madison, Wisconsin). TexasRed-conjugated donkey anti-mouse IgG secondary antibodies for immunofluorescence microscopy were purchased from Jackson ImmunoResearch Laboratories (Pennsylvania).

Expression in Sf9 and COS-1 Cells

For insect cell expression, a synthetic gene encoding bacterio-opsin (Dunn *et al.*, 1987) was cloned into the baculovirus transfer vector pVL1393 (Invitrogen, San Diego, California). Recombinant baculoviruses were generated using the Baculo-gold kit from Pharmingen (San Diego, California). Sf9 cells (Invitrogen) were grown in complete Grace's medium and transfected with the recombinant vectors using standard protocols (Luckow 1990). For COS-1 cell expression, the bacterio-opsin gene was cloned into a modified pMT3 vector (Ferretti *et al.*, 1986). COS-1 cells (1650-CRL, American Type Culture Collection, Rockville, Maryland) were grown to 70–80% confluence in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 10% heat-inactivated fetal calf serum, D-glucose (4.5 g/liter), 100 IU/ml penicillin, 100 μg/ml streptomycin (COS-medium) at 37°C in a humidified 5% CO_2 incubator. For transient transfection using the DEAE-Dextran method (Cullen, 1987), 1–1.5 μg/ml medium of purified plasmid DNA was applied per dish. Bacterio-opsin was expressed at similar levels (~1 μg protein per 10^6 cells) in both Sf9 cells and COS-1 cells.

Immunofluorescence Microscopy

Sf9 cells were grown on poly-L-lysine-coated glass coverslips, infected with viral stock at multiplicities of infection of about 5–10, and incubated for 2 h. The medium was then exchanged with fresh medium, and the cells were incubated for a further 48 h at 27°C. Cycloheximide was then added at a concentration of 100 μg/ml to inhibit protein synthesis and the cells were incubated for a further 2 h period. Cells were prepared for confocal microscopy essentially as described by DeTomaso *et al.* (1993), and incubated with the bR114 monoclonal antibody, followed by incubation with TexasRed-conjugated donkey anti-mouse IgG. COS-1 cells were grown on glass coverslips coated with poly-L-lysine. Sixteen to eighteen hours after transfection, the cells were rinsed with phosphate-buffered saline (PBS, pH 7.2) containing 10 mM glycine and 0.02% azide. The rinsed cells were fixed and permeabilized by methanol (5 min) and acetone treatment (5 min). After blocking with 10% bovine serum albumin (in PBS), the cells were incubated with the bR114 monoclonal antibody (1:3,000 dilution). TexasRed-conjugated donkey anti-mouse IgG (1:400 dilution) was used to visualize the subcellular localization of the expressed bacterio-opsin. Immunofluorescence pictures were taken either with a BIO-RAD MRC 600 confocal imaging system or a Nikon Microphot microscope using a 60× oil immersion lens.

Retinal Reconstitution Experiments

Membrane suspensions from transfected Sf9 cells were prepared by lysing cells in 100 mM $CaCl_2$ followed by sonication for ~1 min using a Branson Sonifier. Membranes were collected by centrifugation at 50,000*g* and resuspended in PBS at pH 7. For the COS-1 cells, an ER enriched microsomal membrane suspension of transiently transfected COS-1 cells was first prepared. To do this, cells were collected in hypotonic buffer, stabilized with sucrose (0.9 M final), and subjected to Dounce homogenization (60 strokes). Cellular debris and nuclei were spun out (at 500*g*) and the supernatant was purified by centrifugation (at 30000*g*) through a 1 M sucrose cushion. The microsomal pellet was resuspended in PBS, pH 7.2. Membrane suspensions from either Sf9 cells or COS-1 cells were divided into two fractions. A freshly prepared ethanolic solution of *all-trans* retinal (final concentration about 1 μM) was added directly to one fraction, and absorption spectra were recorded using the other fraction as a reference. Spectroscopic measurements were carried out at 20°C in a UV-2101 Shimadzu spectrophotometer

equipped with an integrating sphere accessory. Formation of the chromophore was rapid and essentially complete 2–3 min as observed with renatured bacterio-opsin (Braiman *et al.*, 1987).

RESULTS AND DISCUSSION

Bacterio-opsin Does Not Exit the ER when Expressed in Sf9 or COS-1 Cells

The subcellular localization of bacterio-opsin expressed in Sf9 cells was determined by confocal immunofluorescence microscopy. The expressed protein was observed to be present in a broad band extending from the cell surface to the nuclear membrane (Fig. 1A), confirmed by comparing the immunofluorescence image to the image recorded by bright field microscopy. No staining was observed in control experiments with untransfected cells (Fig. 1B). To test whether the broad band reflects localization of the protein in the ER, Sf9 cells were infected with recombinant baculoviruses carrying the gene for the IgG heavy chain. IgG heavy chain expressed in Sf9 insect cells has been previously shown by confocal microscopy to be retained in the ER (DeTomaso *et al.*, 1993). This result is confirmed in Fig. 1C, supporting the conclusion that the majority of bacterio-opsin expressed in Sf9 cells is localized to the ER.

Similar localization experiments were carried out with COS-1 cells transiently transfected with wild-type bacterio-opsin. As in the case of Sf9 cells, the protein localized to a reticular pattern which is typical for the ER (Fig. 2). Since the nuclei of COS-1 cells are much smaller relative to the size of the cell, the ER network is more readily visualized. A similar staining pattern was observed in COS-1 cells that were transiently transfected with either a bacterio-opsin mutant that misfolds, or with a bovine opsin mutant lacking two or more transmembrane segments (Heymann and Subramaniam, unpublished).

Bacterio-opsin Is Correctly Folded in Membranes Isolated from Sf9 and COS-1 Cells

ER localization is often associated with misfolding and aggregation (Gething and Sambrook, 1990). To determine if the expressed protein was folded into a structure similar to that of native bacteriorhodopsin, *all-trans* retinal was added to membranes isolated from Sf9 cells and COS-1 cells expressing bacterio-opsin. Previous reconstitution experiments have established that the formation of functional bacteriorhodopsin can be monitored by the change in λ_{max} of retinal from 380 nm (in the free state) to 560 nm, when it is bound as a protonated Schiff's base to Lys 216 in the seventh transmembrane helix (Oesterhelt and Schuhmann, 1974, Stoeckenius *et al.*, 1979). Figure 3A demonstrates that bacterio-opsin expressed in Sf9 cells bound *all-trans* retinal and formed a chromophore with the characteristic absorption maximum. A similar result was observed upon reconstitution of bacterio-opsin expressed in COS-1 cells with *all-trans* retinal (Fig. 3B). The control experiment in Fig. 3C shows that no chromophore formation is observed upon addition of *all-trans* retinal to membranes isolated from cells expressing a misfolded mutant (Heymann and Subramaniam, unpublished) of bacteriorhodopsin.

CONCLUSION

Folding of plasma membrane proteins is generally initiated in the ER and the accurate completion of this step is believed to be a necessity for entering more distal compartments of the secretory pathway (Hurtley and Helenius, 1989). Thus, ER localization of a protein that is normally not resident in the ER is usually an indication of misfolding, aggregation, or interaction with components of the ER. Our results show that bacterio-opsin can acquire a functional conformation in ER membranes of Sf9 and COS-1 cells upon binding of retinal, and provides a clear-cut example of a protein that is localized in the ER but is not misfolded. Our findings add to the other recently documented examples of imtegral membrane proteins that are also correctly folded when expressed in tissue culture, but do not exit the ER membrane (Ruetz *et al.*, 1993; Pasyk and Foskett, 1995; Goldberg *et al.*, 1995).

Based on our observations and those of others, we propose that membrane proteins that do not exit the ER fall into at least two distinct categories: (i) those which are correctly inserted, integrated, and folded in the ER membrane, and do not exit the ER for reasons other than misfolding, and (ii) those which are improperly folded, and perhaps incorrectly inserted into the ER membrane. It is conceivable that this latter class of proteins may be retained in the ER primarily because of their nonspecific association with important ER-resident membrane proteins. A systematic determination of the extents of association of various heterologously

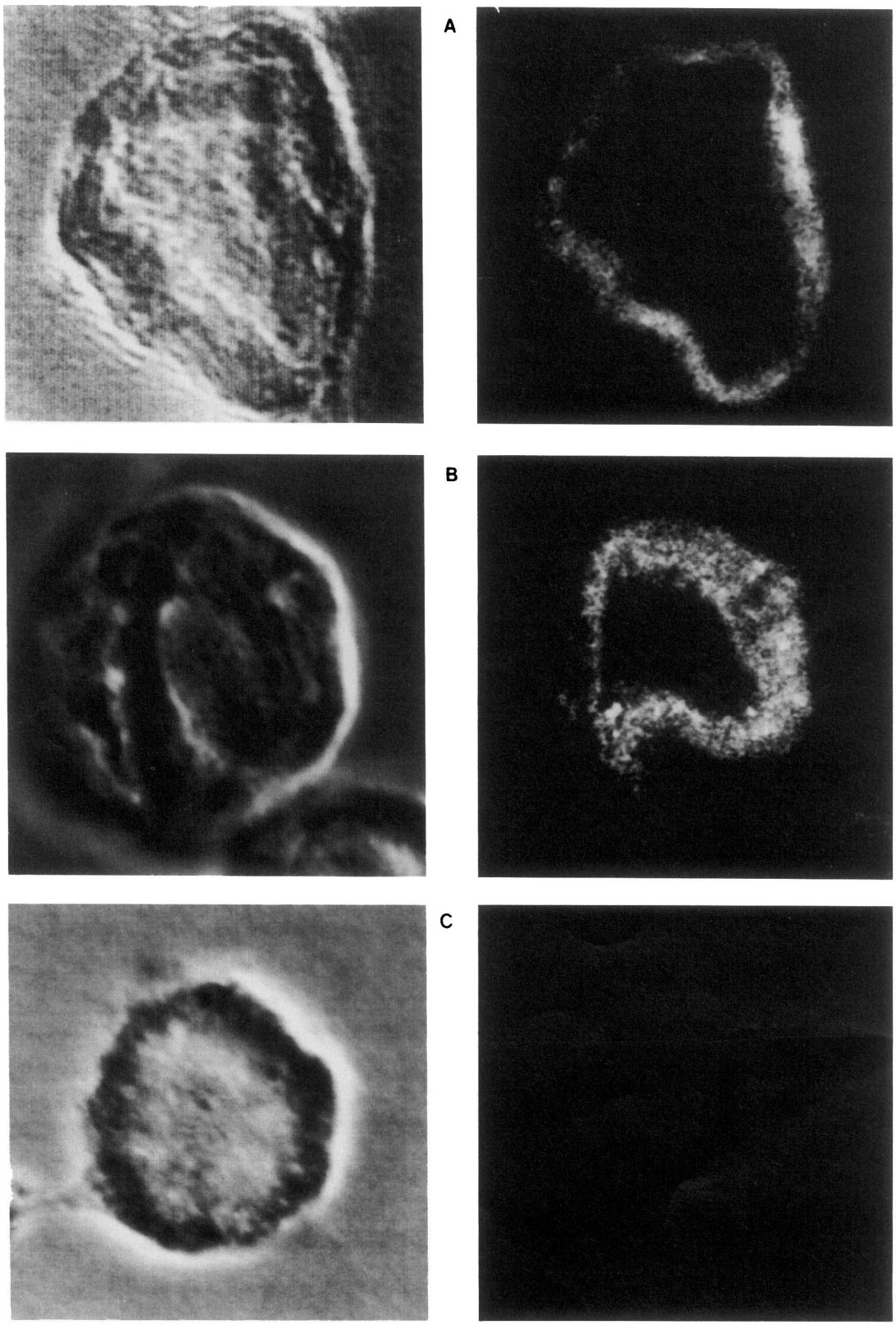

Fig. 1. Bright field (left) and confocal micrographs (right) of Sf9 cells infected with virus containing the gene for wild-type bacteriorhodopsin (A), or the gene for the IgG heavy chain (C). Cells shown in A were treated with bR114 as the primary antibody. Cells in (B) are mock transfected and, processed as in (A). TexasRed-conjugated donkey anti-mouse IgG was used for detection in each case.

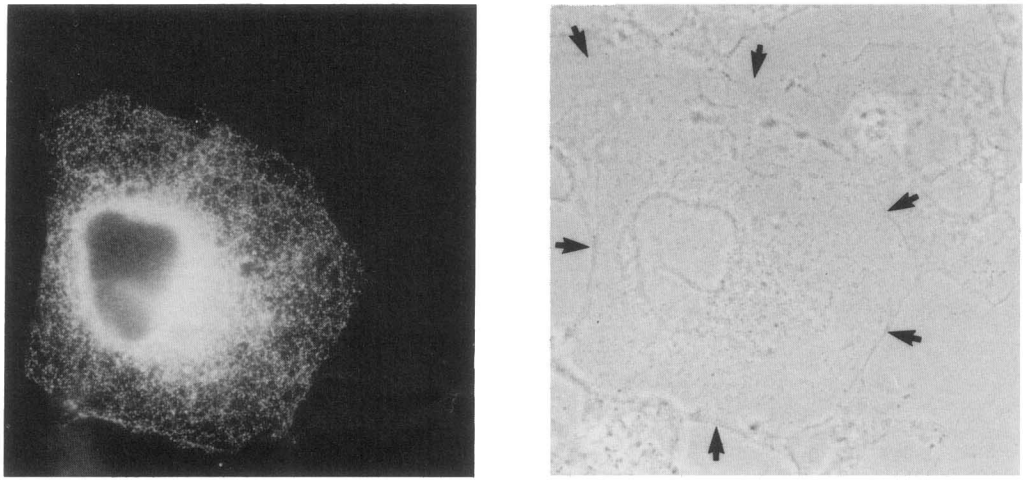

Fig. 2. Bright field (left) and immunofluorescence images (right) of COS-1 cells transiently transfected with wild-type bacterio-opsin visualized with bR114 as in Fig. 1.

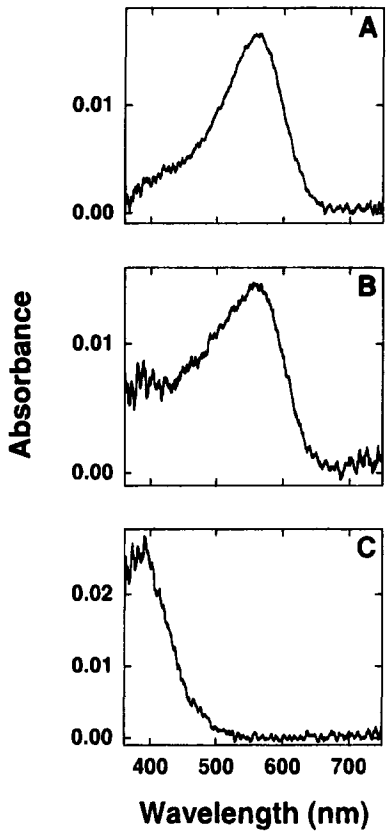

Fig. 3. Chromophore formation in membranes isolated from cells expressing bacterioopsin. A: wild-type bacterio-opsin expressed in Sf9 insect cells, B: wild-type bacterio-opsin expressed in COS-1 cells, C: misfolded mutant of bacteriorhodopsin expressed in Sf9 cells (control).

expressed proteins with different ER-resident proteins is likely to provide a critical evaluation of this hypothesis.

REFERENCES

Braiman, M. S., Stern, L. J., Chao, B. H., and Khorana, H. G. (1987). *J. Biol. Chem.* **262**, 9271–9276.

Cullen, B. R. (1987). *Methods Enzymol.* **152**, 684–704.

DeTomaso, A. W., Xie, Z. J., Liu, G., and Mercer, R. W. (1993). *J. Biol. Chem* **268**, 1470–1478.

Dunn, R., Hackett, N. R., McCoy, J. M., Chao, B. H., Kimura, K., and H. G. Khorana (1987). *J. Biol. Chem.* **262**, 9246–9254.

Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., and Oprian, D. D. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 599–603.

Gething, M.-J. and Sambrook, J. (1990). *Sem. Cell Biol.* **1**, 65–72.

Goldberg, A. F. X., Moritz, O. L., and Molday, R. S. (1995). *Biochemistry* **34**, 14213–14219.

Grisshammer, R., and Tate, C. G. (1995). *Quart. Rev. Biophys.* **28**, 315–422.

Hildebrandt, V., Ramezani-Rad, R., Swida, U., Wrede, P., Grzesiek, S., Primke, M., and Blüdt, G. (1989). *FEBS Lett.* **243**, 137–140.

Hurtley, S. M., and Helenius, A. (1989). *Annu. Rev. Cell. Biol.* **5**, 277–307.

Karnik, S. S., Nassal, M., Doi, T., Jay, E., Sgaramella, V., and Khorana, H. G. (1987). *J. Biol. Chem.* **262**, 9255–9263.

Kimura, K., Mason, T. L., and Khorana, H. G. (1982). *J. Biol. Chem.* **257**, 2859–2867.

Lang-Hinrichs, C., Queck, I., Büldt, G., Stahl, U., and Hildebrandt, V. (1994). *Mol. Gen. Genet.* **244**, 183–188.

Luckow, V. A. (1990). *Recombinant DNA Technology and Applications*, McGraw-Hill, New York.

Nagel, G., Mockel, B., Büldt, G., and Bamberg, E. (1995). *FEBS Lett.* **377**, 263–266.

Oesterhelt, D., and Schuhmann, L. (1974). *FEBS Lett.* **44**, 262–265.

Pasyk, E. A., and Foskett, J. K. (1995). *J. Biol. Chem.* **270**, 12347–12350.

Ruetz, S., Lindsey, A. E., Ward, C. L., and Kopito, R. R. (1993). *J. Cell Biol.* **121**, 37–48.

Sonar, S., Patel, N., Fischer, W., and Rothschild, K. J. (1993). *Biochemistry* **32**, 13777–13781.

Stoeckenius, W., Lozier, R., and Bogomolni, R. (1979). *Biochim. Biophys. Acta* **505**, 215–278.