Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6006–6009, July 1992
Cell Biology

Folate receptor allows cells to grow in low concentrations of 5-methyltetrahydrofolate

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Communicated by A. J. Hudspeth, April 9, 1992 (received for review February 26, 1992)

ABSTRACT **The folate receptor mediates the uptake of 5-methyltetrahydrofolate in certain cultured cells by a process called potocytosis. When these cells are grown in physiological concentrations of folate, the receptor increases the efficiency of vitamin uptake by 30-fold. We now show that PAM 212 cells, a mouse keratinocyte cell line, are unable to grow in 1 nM 5-methyltetrahydrofolate unless they express a functional folate receptor. These results suggest that under certain conditions, tissue cells in the body may depend on the folate receptor to obtain enough 5-methyltetrahydrofolate for growth.**

Potocytosis is a high-affinity uptake process that cells use to obtain essential, low molecular weight molecules (1) from their environment. Membrane-bound proteins at the cell surface concentrate the molecules within closed caveolae. Once the molecules are released into the caveolar space, they diffuse into the cytoplasm through carrier proteins in the membrane.

Caveolae are the membrane-bound transport vehicles for potocytosis (1). This membrane specialization is present on the surface of a wide variety of cells (2, 3) but has been studied most thoroughly in endothelial cells (4–6). In these cells, caveolae form vesicles or open channels that appear to transport molecules from the blood to the tissue spaces. The invaginated morphology of caveolae makes them easy to recognize in thin-section electron micrographs (7). Rapid-freeze, deep-etch images have shown that the cytoplasmic surface of invaginated and flat caveolae membranes is decorated with a characteristic striated coat. This coat is resistant to removal with high salt (7). A protein component of the coat has recently been identified and named caveolin (7). The membrane coat may play a role in controlling the closing or pinching off of caveolae to form the transport compartment for potocytosis.

Potocytosis was discovered by studying the receptor-mediated uptake of 5-methyltetrahydrofolate in folate-depleted MA104 cells (8–12). The folate receptor is a glycosyl-phosphatidylinositol-anchored membrane protein that cycles in and out of the cell by caveolae. Each cycle delivers a quantity of the vitamin to the interior of closed caveolae, where it dissociates from the receptor and diffuses through anion carriers in the membrane into the cytoplasm. When cells are grown in physiological concentrations of 5-methyltetrahydrofolate, the receptor increases the efficiency of vitamin uptake by 30-fold (9). It is not known whether this increased efficiency confers any survival advantage on cells growing under physiological conditions. If it does, then cells cultured in the presence of 1–10 nM 5-methyltetrahydrofolate should grow more rapidly if they express the folate receptor. The availability of the cDNA for the folate receptor (13) made it possible to test this hypothesis.

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MATERIALS AND METHODS

Cell Culture. Transfected cell lines were maintained in cDMEM (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum), which contains 2.3 μM folic acid. Before receptor activity was measured, cells were grown for 5 days in folate-free RPMI 1640 medium supplemented with 10% fetal bovine serum (5-methyltetrahydrofolate concentration, <1 nM) to deplete the cytoplasm and receptors of endogenous folate.

Vector Construction. The plasmid pJB20 contains the cytomegalovirus promoter upstream of a polycloning site. The simian virus 40 T-antigen intron sequence has been inserted into the *Hin*dIII–*Bam*HI region of the polycloning site. This is followed by the human growth hormone transcription termination signal and a simian virus 40 promoter/enhancer driving the cDNA for neomycin resistance (14). To construct the vector containing the folate receptor cDNA (designated FRwt), the polycloning site was opened with *Eco*RI and the folate receptor cDNA was inserted (15). The orientation was confirmed by restriction mapping and sequencing.

Transfection. PAM 212 cells (16) were plated onto 100-mm dishes (1.5×10^5 cells per dish) and cultured in 10 ml of cDMEM containing Polybrene at 3 mg/ml (15). After 18 hr, the medium was replaced with 3 ml of cDMEM containing Polybrene at 3 mg/ml. A 100-ng sample of either the vector plasmid (pJB20) or the folate receptor plasmid (FRwt) was mixed with the medium before the Polybrene was added, and the mixture was added to separate plates of cells and incubated for 6 hr at 37°C. The DNA was removed, 30% (vol/vol) dimethyl sulfoxide in cDMEM was added, and the cells were cultured for 24 hr. The medium was changed to cDMEM containing the neomycin analogue G418 at 1 mg/ml. On day 5 after the transfection, the medium was replaced with fresh selection medium to remove dead cells. By approximately day 14, individual colonies could be identified. These were selected and expanded. Clones of transfected cells containing either pJB20 (designated Vector-PAM 212) or FRwt (designated FR-PAM 212) were isolated by limiting dilution.

Binding Studies. Folate-depleted cells (see *Cell Culture*) were washed once with Dulbecco's phosphate-buffered saline and incubated in the presence of 10 nM [^{3}H]folic acid in folate-free RPMI 1640 for 2 hr at 37°C to saturate external and internal binding sites (11). The cells were then washed twice with phosphate-buffered saline and washed for 30 sec with 150 mM NaCl at pH 3 to remove [^{3}H]folate from external receptors. After acid treatment, the cells were removed from the dish with trypsin and the amount of internalized [^{3}H]folic acid was measured by scintillation counting.

Immunofluorescence. Cells were cultured in cDMEM until confluent. The cells were chilled to 4°C and incubated for 1 hr at 4°C with either monoclonal anti-folate receptor IgG or

Abbreviation: cDMEM, Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

an irrelevant monoclonal IgG according to a standard protocol (8). The cells were then washed and processed to localize the bound IgG by indirect immunofluorescence.

[^{3}H]Thymidine Incorporation. FR-PAM 212 cells and Vector-PAM 212 cells were precultured in either folate-rich or folate-free RPMI 1640 supplemented with 10% fetal bovine serum. After 7 days of preculture, the cells were plated at a density of 2×10^4 cells per well into individual wells of a 96-well plate and cultured for the indicated time in folate-free RPMI 1640 supplemented with 10% fetal bovine serum. On the indicated day, the ability of the cells to incorporate [^{3}H]thymidine was measured in triplicate samples by adding 1 μCi (37 kBq) of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) to each well and incubating the cells an additional 16 hr. The cells were then trypsinized and harvested for liquid scintillation counting.

Other Methods. Protein was determined by the method of Bradford (17).

RESULTS

PAM 212 cells, a mouse keratinocyte cell line, were transfected with either the vector (Vector-PAM 212 cells) or the vector containing the cDNA for the folate receptor (FR-PAM 212 cells). Isolates were assayed for the presence of the folate receptor by indirect immunofluorescence. FR-PAM 212 cells (Fig. 1*B*) incubated with monoclonal anti-folate receptor IgG (18) had variable numbers of bright foci scattered across the surface of each cell. At higher magnification (Fig. 2), IgG binding sites were seen to be organized into discrete clusters that had an appearance identical to that of native receptor clusters present on the surface of MA104 cells (8). Vector-PAM 212 cells did not stain with anti-folate receptor IgG (Fig. 1*D*) and neither set of cells stained with an irrelevant monoclonal antibody (Fig. 1 *A* and *C*).

The ability of the two transfected cell lines and the nontransfected, control cells to bind and internalize [^{3}H]folic acid is shown in Table 1. Vector-PAM 212 cells and PAM 212 cells did not bind [^{3}H]folic acid. The amount internalized by these two cells also was not above background. By contrast, FR-PAM 212 cells bound on their surface 1.1 pmol per 10^6 cells and internalized 0.35 pmol per 10^6 cells. Folic acid is a high-affinity folate that remains bound during receptor internalization and receptor recycling (11). Therefore, these results indicate that FR-PAM 212 cells express functional receptors that can be internalized.

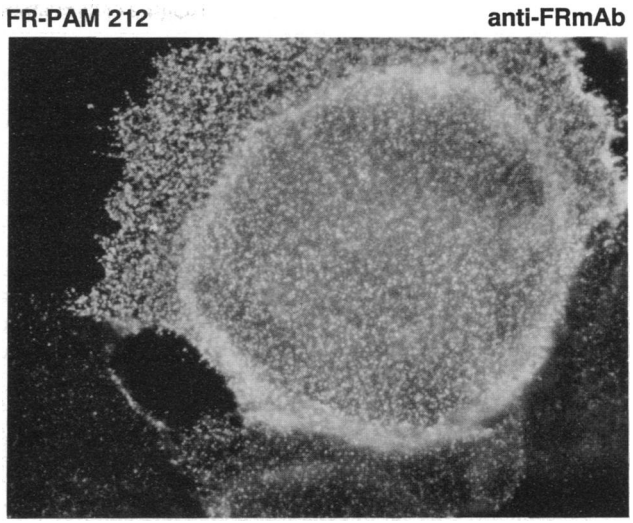

FIG. 2. High-magnification view of FR-PAM 212 cells stained with monoclonal anti-folate receptor IgG. Cells were prepared and processed exactly as described in the legend to Fig. 1. (×4865.)

Cells obtain 5-methyltetrahydrofolate by potocytosis when they are made folate-deficient (12). Therefore, receptor-dependent cell growth should be manifested in cells that have been depleted of folate for several days. FR-PAM 212 cells and Vector-PAM 212 cells were cultured in the presence or absence of 2.3 μM folate for 7 days (Fig. 3). Each set of cells was harvested, plated at either 0.5, 1, or 2×10^4 cells per dish, and cultured for an additional 5 days in folate-free medium containing 10% fetal bovine serum as the only source of 5-methyltetrahydrofolate. This amount of serum raises the folate concentration in the medium to ≈1 nM (12). Both sets of cells grew well when they were precultured in high-folate

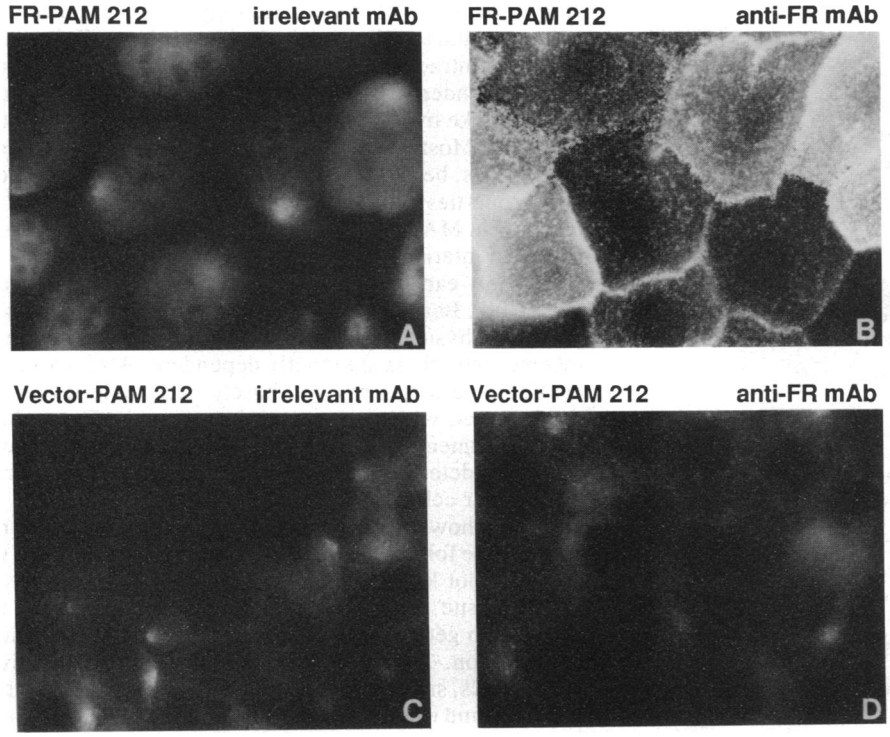

FIG. 1. Immunofluorescence localization of the folate receptor in PAM 212 cells transfected with either the vector (*C* and *D*) or the vector containing the full-length cDNA for the folate receptor (*A* and *B*). Isolates were cultured in cDMEM until confluent. The cells were chilled to 4°C and incubated for 1 hr at 4°C with either monoclonal anti-folate receptor IgG (*B* and *D*) or an irrelevant monoclonal IgG (*A* and *C*) according to a standard protocol (8). The cells were then washed and processed to localize the bound IgG by indirect immunofluorescence. mAb, Monoclonal antibody. (×1000.)

Table 1. Binding of [^{3}H]folic acid to transfected PAM 212 cells

Cell line	[^{3}H]Folic acid, pmol per 10^6 cells	
	Surface	Internalized
PAM 212	0.00	0.04
Vector-PAM 212	0.00	0.06
FR-PAM 212	1.10	0.35

medium (Fig. 3*A*, day 5). By contrast, only the cells that expressed the folate receptor (FR-PAM 212) were able to grow when the cells were precultured in low-folate medium (Fig. 3*B*, day 5). This growth advantage was expressed regardless of the initial number of cells plated in the dish.

A more quantitative method for measuring cell growth is [^{3}H]thymidine incorporation. Two sets of transfected cells (FR-PAM 212 and Vector-PAM 212) were maintained in 2.3 μM folate (Fig. 4, maintenance culture) until the beginning of the experiment. One set of cells was plated and cultured for 7 days in high-folate medium (Fig. 4*A*, preculture), and the other set was cultured for the same period in low folate medium (Fig. 4*B*, preculture). On day 8, both sets of cells were transferred to medium containing 1 nM 5-methyltetrahydrofolate and cultured for 5 days. This made the total time the cells were in low-folate medium either 5 days (Fig. 4*A*) or 12 days (Fig. 4*B*). [^{3}H]Thymidine incorporation was measured on each day of the last 5 days in culture. Both the FR-PAM 212 cells and Vector-PAM 212 cells cultured in high-folate medium incorporated increasing amounts of [^{3}H]thymidine on successive days of culture (Fig. 4*A*), indicating that cell growth was unimpaired. By contrast, only FR-PAM 212 cells incorporated [^{3}H]thymidine if the two sets of cells were precultured in low-folate medium (Fig. 4*B*). After 12 days in low-folate medium, the FR-PAM 212 cells incorporated ≈9-fold more [^{3}H]thymidine than Vector-PAM 212 cells.

DISCUSSION

These experiments define conditions where growth of PAM 212 cells is dependent on the folate receptor. The essential step is lowering the 5-methyltetrahydrofolate concentration in the medium to subphysiological levels for sufficient time to make the intracellular concentration of 5-methyltetrahydrofolate dependent on the receptor. The increased efficiency of folate uptake by the receptor confers a growth advantage on the cells. Most likely the vitamin is being internalized by potocytosis, because the transfected receptor displayed several properties previously described for the natural folate receptor in MA104 cells: a clustered organization (8) and an ability to internalize and recycle folic acid (11).

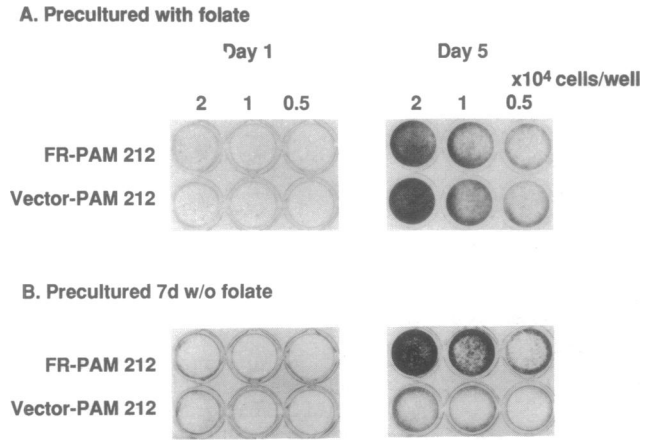

FIG. 3. Folate receptor-dependent growth of transfected PAM 212 cells. FR-PAM 212 cells and Vector-PAM 212 cells were precultured for 7 days in either folate-rich (*A*) or folate-free (*B*) RPMI 1640 supplemented with 10% fetal bovine serum. The folate concentration in the first medium was 2.3 μM and in the second medium ≈1 nM. Both sets of cells were harvested and plated at densities of 0.5, 1, or 2 × 10^4 cells per well in 24-well plates and cultured for 1 day or 5 days in folate-free RPMI 1640 medium containing 10% fetal bovine serum. On days 1 and 5 the cells were washed once with phosphate-buffered saline and stained with 1% crystal violet in absolute methanol for 30 min at room temperature. Following staining, the cells were washed several times with distilled water and allowed to dry before photography.

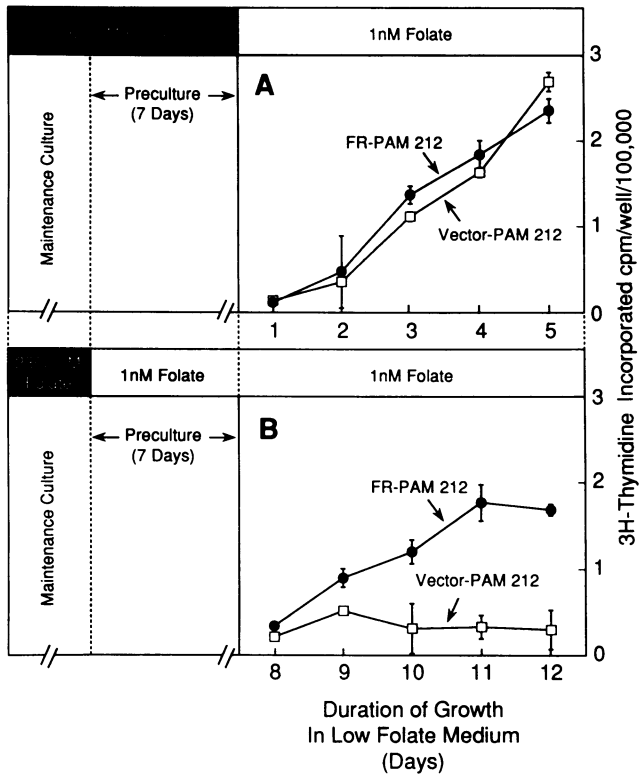

FIG. 4. Folate receptor-dependent incorporation of [^{3}H]thymidine in transfected PAM 212 cells. FR-PAM 212 cells (●) and Vector-PAM 212 cells (□) were precultured in either high-folate medium (*A*) or low-folate medium (*B*) exactly as described in the legend to Fig. 3. After 7 days of preculture, the cells were plated at a density of 2 × 10^4 cells per well into individual wells of a 96-well plate and cultured for the indicated time in folate-free RPMI 1640 supplemented with 10% fetal bovine serum. On the indicated day, the ability of the cells to incorporate [^{3}H]thymidine was measured in triplicate samples by adding 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol) to each well and incubating the cells an additional 16 hr. The cells were then trypsinized and harvested for liquid scintillation counting. Each bar indicates the standard error of the mean.

Probably each cell type has a different folate requirement for growth. Based on this demand, some cells may be able to grow in physiological 5-methyltetrahydrofolate without a receptor but others are strictly dependent. Also, certain cells may be able to get by with barely detectable levels of the receptor yet, with no receptor, have impaired growth. Further experiments using the transfection assay system will be needed to determine the minimum number of receptors that a particular cell needs to grow.

Exactly how the folate requirements of a tissue culture cell relate to the folate demands of a similar type cell in the whole animal is not known. The results of this study suggest that certain tissue cells in the body may depend on the folate receptor to get enough 5-methyltetrahydrofolate to support cell division. Likely candidate cells are rapidly dividing normal cells, such as gut epithelium; diseased epithelial cells, such as found in psoriatic skin; and malignant cells. This may

explain why several tumor cell lines express high concentrations of the folate receptor (18). The influence of this receptor on the growth potential of diseased cells is an important area for future investigation.

The folate receptor must have other functions besides providing for optimal growth conditions in select cells. The tissues with the highest concentrations of the receptor are kidney and choroid plexus (19). These two organs are involved in retrieving and moving substances from one tissue space to another. Presumably the folate receptor provides a high-affinity mechanism that increases the efficiency of vectorial folate transport in these tissues. Molecular techniques make it possible to design experiments that will elucidate the function of the folate receptor in different tissue settings.

This study would not have been possible without the generous help of David W. Russell, who provided the pCMV1 vector from which the pJB20 vector was derived; Colleen Brewer and Pam Beck, who constructed the pJB20 vector from the pCMV1 vector; and Paul Bergstresser, who provided space, facilities, and expertise to carry out many of these studies. This work was supported by the Texas Affiliate of the American Heart Association (90G-086), American Cancer Society Individual Institutional Grant IN-1421, and the National Institutes of Health (GM43169, AR40042, CA52625, DK01860).

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