Internalization of the opioid growth factor, [Met$^5$]-enkephalin, is dependent on clathrin-mediated endocytosis for downregulation of cell proliferation

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Cheng F, McLaughlin PJ, Banks WA, Zagon IS. Internalization of the opioid growth factor, [Met$^5$]-enkephalin, is dependent on clathrin-mediated endocytosis for downregulation of cell proliferation. Am J Physiol Regul Integr Comp Physiol 299: R774–R785, 2010. First published June 30, 2010; doi:10.1152/ajpregu.00318.2010.—The opioid growth factor (OGF; [Met$^5$]-enkephalin), a constitutively expressed and tonically active inhibitory peptide, interacts with the OGF receptor (OGFr) to form an endogenous growth-regulating pathway in homeostasis. Amplification of OGF-OGFr interfacing in animal and clinical studies depresses development, neoplasia, angiogenesis, and immunity. Disruption of the OGF-OGFr axis accelerates cell proliferation and has been particularly important in wound repair. To investigate how OGF enters cells, OGF was labeled with 5,6-tetramethylrhodamine OGF (RhoOGF) to study its uptake in live cells. African green monkey kidney cells (COS-7) incubated with RhoOGF exhibited a temperature-dependent course of entry, being internalized at 37°C but not at 4°C. RhoOGF was detected in the cytoplasm 15 min after initial exposure, observed in both cytoplasm and nucleus within 30 min, and remained in the cells for as long as 5 h. A 100-fold excess of OGF or the opioid antagonist naltrexone, but not other opioid ligands (some selective for classic opioid receptors), markedly reduced entry of RhoOGF into cells. RhoOGF was functional because DNA synthesis in cells incubated with RhoOGF ($10^{-8}$ to $10^{-6}$ M) was decreased 24–36%, and was comparable to cells treated with unlabeled OGF (reductions of 26–39%). OGF internalization was dependent on clathrin-mediated endocytosis, with addition of clathrin siRNA diminishing the uptake of RhoOGF and upregulating DNA synthesis. RhoOGF clathrin-mediated endocytosis was unrelated to endosomal or Golgi pathways. Taken together, these results suggest that OGF enters cells by active transport in a saturable manner that requires clathrin-mediated endocytosis.

human mesenchymal stem cells; active transport; DNA synthase; nucleocytosomal transport; siRNA

THE PLASMA MEMBRANE FORMS a delicate boundary between the extracellular milieu and intracellular constituents. Cells internalize extracellular material (e.g., ligands, soluble molecules, proteins) to regulate homeostatic processes. Endocytosis is the process by which cells absorb molecules through several routes, including clathrin-coated pits and vesicles, caveolae, lipid rafts, macropinosis, and phagocytosis (7–10, 12, 19).

Endogenous opioid peptides are important in neurotransmission/neuromodulation (1, 22) as well as serving in other functions, such as regulating DNA synthesis and cell proliferation (2–6, 13, 18, 21, 23–25, 30). One native opioid peptide, [Met$^5$]-enkephalin, has been recognized as vital to the maintenance of cell proliferation and, because of its distinct location and function in neural and nonneural cells, has been termed the opioid growth factor (OGF) to distinguish its unique biological role. OGF is a pentapeptide of 574 mol wt that is derived from preproenkephalin by differential processing. This peptide is known to be secreted by cells, and to act in an autocrine/paracrine manner to inhibit DNA synthesis by delaying the G1/S interface of the cell cycle through modulation of the cyclin-dependent kinase inhibitor pathways. OGF action is mediated by the OGF receptor (OGFr), located on the outer nuclear envelope. Following binding, the OGF-OGFr complex translocates into the paranuclear cytoplasm and undergoes nucleocytosomal transport that is dependent on nuclear localization signaling, karyopherin β, and Ras-related nuclear protein (Ran).

OGF action both in vitro and in vivo, is extremely rapid. For example, DNA synthesis can be depressed within 2 to 3 h of OGF administration (6, 26, 27). However, the mechanism of entry of OGF into cells to modulate cell proliferative processes is unknown. The present investigation takes advantage of a fluorescent labeled OGF in the C5/C6 position [5,6-tetramethylrhodamine-Tyr-Gly-Gly-Phe-Met]. The cellular location of rhodamine-labeled OGF (RhoOGF) can be visualized in live cells by fluorescent microscopy. Our strategy was to employ a cell line, African green monkey kidney cells (COS-7), which does not have classic opioid receptors but does contain OGFr and a functioning OGF-OGFr system to examine the entry of RhoOGF into cells (31). Moreover, COS-7 cells lack endogenous caveolin-1 (14, 15, 20), thereby eliminating one major pathway of peptide trafficking. The ubiquity of OGF passage into cells was assessed in two additional human cell lines representing a mesenchymal stem cell and a cancer cell line known to express both classic and OGFr opioid receptors. To address the question of whether the rhodamine probe provided a similar function as OGF in depressing cell proliferation, DNA synthesis of COS-7 cells exposed to RhoOGF or OGF was ascertained. Finally, a series of experiments were performed to identify the specific pathway of endocytosis utilized by OGF for entry into the cells.

MATERIALS AND METHODS

Materials

Cell culture. COS-7 cells, human mesenchymal stem cells (hMSCs), and human ovarian cancer cells (SKOV-3) were utilized in these studies. COS-7 and SKOV-3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). hMSCs were a gift from Dr. H. Donahue (Pennsylvania State University, Hershey, PA). COS-7 cells were grown in DMEM, SKOV-3 cells were cultured in RPMI, and human mesenchymal stem cells (hMSCs) were grown in base medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were grown at 37°C in a humidified 5% CO2 atmosphere.

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Fig. 1. Time course of internalization of rhodamine (Rho) opioid growth factor (RhoOGF) into African green monkey kidney cells (COS-7). COS-7 cells were incubated at 37°C with RhoOGF for 5, 10, 15, 30, or 60 min (A), as well as 3, 5, and 7 h (B) after removal of RhoOGF following a 1-h incubation. Cells were stained with Hoechst and analyzed by epifluorescent (Epi-F) (RhoOGF or Hoechst) or differential interference contrast (DIC) microscopy. Within 15 min of the addition of RhoOGF, cytoplasmic staining with enrichment of fluorescent signal was observed and at 30-min a signal was noted in the nucleus. RhoOGF remained in cells for up to 5 h and was no longer present at 7 h. Bar = 10 μm.
in RPMI, and hMSC cells were incubated in low-glucose DMEM (GIBCO, Carlsbad, CA); all media were supplemented with 10% fetal bovine serum, 1.2% sodium bicarbonate, and 5,000 U/ml penicillin, 5 μg/ml streptomycin, and 10 μg/ml neomycin. Cell cultures were maintained in a humidified atmosphere of 5% CO2-95% air at 37°C, unless otherwise noted.

Reagents. The following compounds were obtained from the indicated sources. RhoOGF (purity 95.17%) was synthesized by BIO WORLD (Dublin, OH). [Met5]-enkephalin (OGF), naltrexone (NTX), [α-Pen2,5]-enkephalin, [α-Ala2, MePhe4, Glyol5]-enkephalin, U69593, rhodamine, and rhodamine-dextran (70,000 mol wt; RD70S) were purchased from Sigma (St. Louis, MO). Hoechst 33258 and transferrin Alexa Fluor 488 conjugate were obtained from Molecular Probes (Eugene, OR). RhoOGF was dissolved in 5% DMSO (Me2SO) and PBS and used at a final concentration of 10−5 M. RD70S and rhodamine were dissolved in 100% ethanol and used at a final concentration of 10−5 M. OGF, NTX, [α-Ala2, MePhe4, Glyol5]-enkephalin (DAMGO), [α-Pen2,5]-enkephalin (DPDPE), and U69593 were dissolved in sterile water and used at a final concentration of 10−3 M for competitive studies. Monodansylcadaverine (MDC) and EIPA were purchased from Sigma, and methyl-β-cyclodextrin (MβCD) was kindly provided by Dr. Schengrund from The Pennsylvania State University.

Methods

Each experiment was conducted three independent times.

RhoOGF uptake studies. For experiments on RhoOGF uptake, cells were grown to 50–80% confluence in 35-mm glass bottom culture dishes (MatTek, Ashland, MA). To study intracellular RhoOGF localization, cells were preincubated in serum-free medium for 30 min, and then 10−5 M RhoOGF was added into living cell cultures for 5, 15, 30, or 60 min. Before washing with PBS, cells were stained with Hoechst (50 ng/ml) for 5 min. Subsequently, cells were washed 5 times with PBS, and analyzed by epifluorescent or differential contrast interference (DIC) microscopy with an IX81 Olympus inverted microscope (Center Valley, PA). Some images were deconvolved using the constrained iterative algorithm in Slide Book (Intelligent Imaging Innovations, Denver, CO) at ×40 magnification. For each specimen, Z stacks of 12 images were obtained with each image taken 0.5 μm apart. In addition, cell cultures were analyzed by fluorescent microscopy after 3, 5, or 7 h following removal of RhoOGF.

To study the effect of low temperature on the cellular internalization of RhoOGF, cells were preincubated in serum-free medium for 1 h at 4°C or 37°C. RhoOGF was added to each culture for 30 min, followed by staining with Hoechst for 5 min.
For competitive study with other opioids, cells were preincubated with 10^{-3} M OGF, NTX, DAMGO, DPDPE, or U69593 in serum-free medium for 30 min at 37°C. RhoOGF (10^{-5} M) was added to the cultures for 30 min, followed by staining with Hoechst for 5 min.

**5-Bromo-2'-deoxyuridine incorporation study.** To determine DNA synthesis, 5-bromo-2'-deoxyuridine (BrdU) was utilized. COS-7 cells growing on 22-mm coverslips were incubated with OGF, RhoOGF at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M, or rhodamine alone (10^{-5} M) for 24 h. Fresh, complete DMEM medium with 30 μM BrdU (Sigma) was added to each culture for 3 h, and cells were stained according to Donahue et al. (6). Cells were counterstained with DAPI (1 μg/ml) and sealed onto glass slides. At least 100 cells/treatment were counted in a masked fashion for each experiment.

To evaluate the role of clathrin and DNA synthesis, BrdU was utilized. COS-7 cells growing on 22-mm coverslips were transfected with clathrin siRNA for 48 h, followed by the addition of OGF for 24 h. Fresh, complete DMEM medium with 30 μM BrdU (Sigma) was added to each culture for 3 h before staining (6).

**Incubation with inhibitors of endocytosis.** Cells were preincubated with serum-free medium for 30 min at 37°C, and then treated with MDC (300 μM) for 1 h, EIPA (100 μM) for 30 min, MβCD (5 mM) for 30 min, or both MDC (300 μM) and EIPA (100 μM) for 30 min. RhoOGF (10^{-5} M) and transferrin Alexa Fluor 488 conjugate (10 μg/ml) were added to the cell cultures for 30 min prior to staining with Hoechst for 5 min.

**Clathrin siRNA transfection.** Clathrin-targeted siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and scrambled siRNA was purchased from Ambion (Austin, TX). Then 2×10^{5} cells per well were seeded in 35-mm glass-bottom culture dishes containing 1 ml of serum-containing media without antibiotics. Cells were transfected with either 20 nM siRNA with oligofectamine reagent (Invitrogen, Carlsbad, CA) in serum- and antibiotic-free medium. At 24 h, 1 ml of fresh serum-containing media without antibiotics was added to the cultures, and cells were examined at 72 h.

**Western blot analysis.** siRNA transfected cells were solubilized in 100 μl RIPA buffer (1× PBS, 10 μM IGEPAL, 1 mg/ml SDS), containing one complete miniprotease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Total protein concentrations were measured using the DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (40 μg) were subjected to 12% SDS-PAGE followed by transfer of proteins onto polyvinylidene difluoride (Milipore, Billerica, MA) using standard protocols. Membranes were stained with Hoechst and analyzed by Epi-F or DIC microscopy. Bar = 10 μm.
probed with clathrin antibodies (1:200; Santa Cruz Biotechnology) or antibody to β-actin (1:1,000; Sigma) for normalization of protein loading. Blots were washed and reprobed with secondary anti-mouse horseradish peroxidase-conjugated antibodies (GE Healthcare-Amersham Biosciences, Piscataway, NJ), followed by detection with chemiluminescence (GE Healthcare-Amersham).

To quantify expression levels, the optical density of each band was determined by densitometry and analyzed by QuickOne (Bio-Rad Laboratories, Hercules, CA). Each value was normalized to β-actin from the same blot.

RhoOGF and its relationship to endosomes and the Golgi apparatus. COS-7 cells were grown to 50–80% confluence on coverslips in 24-well plates. To study the localization of endosomes and the Golgi apparatus with RhoOGF, cells were preincubated in serum-free media for 30 min prior to the addition of $10^{-5}$ M RhoOGF for 15, 60, or 120 min. Cells were fixed in fresh 2% paraformaldehyde and blocked in 4% BSA. Cells were stained with anti-EEA1 IgG (1:200) to detect early endosomes or anti-GM130 IgG (1:200) to localize the Golgi apparatus, followed by secondary antibodies (Alex Fluor 488 conjugated anti-mouse, InVitrogen, 1:1,000). The preparations were analyzed by epifluorescent or differential interference (DIC) microscopy.

**Statistical analysis.** Data for BrdU incorporation were evaluated using Student’s two-tailed t-test or ANOVA with subsequent comparisons made using Newman-Keuls tests.

**RESULTS**

RhoOGF is internalized in COS-7 cells. To study the entry of OGF into live cells, RhoOGF was added to COS-7 cultures. RhoOGF was internalized as early as 15 min and could be observed in both the cytoplasm and the nucleus of a few cells at 30 min (Fig. 1A). The majority of cells contained RhoOGF in the cytoplasm at 30 min, and generally it took 2 h before most cells had RhoOGF in the nucleus (Fig. 1B). Examination of the spatiotemporal course of RhoOGF in COS-7 cells revealed that RhoOGF remained in cells for as long as 5 h, but...
was no longer detected at 7 h (Fig. 1B). Investigation of cells with deconvolution microscopy confirmed our observations with epifluorescent microscopy, with RhoOGF localized in both the cytoplasm and the nucleus (Fig. 2).

**Entry of RhoOGF into cells is an energy-dependent process.** To determine whether the uptake of RhoOGF into cells is energy dependent, RhoOGF was added to COS-7 cells incubated at 4°C. The result shows that at 4°C, in contrast to cultures at 37°C, no RhoOGF was observed in the cytoplasm or nucleus (Fig. 3).

**Cellular uptake of rhodamine or rhodamine-labeled dextran differs from cell uptake of RhoOGF.** To test the possibility that the fluorescent staining by RhoOGF was due to penetration of the rhodamine tag, COS-7 cells were treated with either a 70-kDa rhodamine-labeled dextran (RD70S) or rhodamine alone at $10^{-5}$ M for 30 min. In RD70S treated cells, no fluorescence was detected (Fig. 4). Cells treated with rhodamine only exhibited a weak signal in the cytoplasm only. In cells exposed to RhoOGF, marked fluorescence was noted in both the cytoplasm and the nucleus.

**Fig. 6. RhoOGF decreased cell proliferation in COS-7 cells.** Cells were incubated with OGF, RhoOGF, or Rho alone at $10^{-5}$, $10^{-6}$, $10^{-7}$, or $10^{-8}$ M for 24 h, and 30 μM 5-bromo-2'-deoxyuridine (BrdU) was added to each culture for 3 h. Bars (means ± SE) represent the percentage of COS-7 cells that incorporated BrdU. Data was statistically different from the control group at **$P < 0.01$ and ***$P < 0.001$.**

**Fig. 7. Internalization of RhoOGF is a ubiquitous feature in human and animal cells.** Human mesenchymal stem cells (hMSCs) and human ovarian cancer cells (SKOV-3) were incubated with RhoOGF for 30 min. Cells were stained with Hoechst and analyzed by Epi-F (RhoOGF or Hoechst) or DIC microscopy. Bar = 10 μm.
**RhoOGF entering cells is peptide specific.** To examine whether other opioid peptides enter cells like RhoOGF, COS-7 cells were treated with 100 times ($10^{-3}$ M) greater concentration of DAMGO, DPDPE, or U69593 to compete with RhoOGF ($10^{-5}$ M). Addition of DAMGO, DPDPE, or U69593 failed to displace the passage of RhoOGF into cells (Fig. 5). However, RhoOGF localization was significantly diminished in cells treated with OGF or NTX.

**RhoOGF downregulates DNA synthesis.** To determine whether RhoOGF modulates DNA synthesis in cells in a manner similar to that of OGF, we examined DNA synthesis after incubation for 24 h with different concentrations of RhoOGF or OGF (Fig. 6). Cells subjected to RhoOGF were decreased by 24–36% from vehicle-treated controls, whereas cells treated with unlabeled OGF were decreased by 26–38%.

Cells exposed to rhodamine alone for 24 h had no change in DNA synthesis from controls.

**Trafficking of RhoOGF is an ubiquitous feature in human and animal cells.** SKOV-3 and hMSC cells treated with RhoOGF for 30 min were observed to contain fluorescent staining in both the cytoplasm and the nucleus (Fig. 7).

**RhoOGF uptake is impaired by inhibitors targeting different endocytic pathways.** To investigate the possible involvement of different endocytic pathways in the cellular uptake of RhoOGF, we tested the effect of MDC (a known inhibitor of clathrin-mediated endocytosis), EIPA (an inhibitor of macropinocytosis), and MβCD (depletes membrane cholesterol and thereby inhibits pathways dependent on caveolae/lipid rafts, as well as clathrin-mediated endocytosis). MDC, EIPA and MDC, and MβCD inhibited RhoOGF uptake (Fig. 8). However, RhoOGF was ob-

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**Fig. 8. RhoOGF uptake is impaired by inhibitors targeting different endocytic pathways.** Cells were preincubated with serum-free medium for 30 min at 37°C, and treated with monodansylcadaverine (MDC) for 1 h, EIPA for 30 min, methyl-β-cyclodextrin (MβCD) for 30 min, or both MDC and EIPA together for 30 min; some cells remained untreated [control (Ctrl)]. Both RhoOGF ($10^{-5}$ M) and transferrin (Tf; 10 µg/ml) were added to cell cultures for 30 min in the presence of inhibitors. Cells were stained with Hoechst and analyzed by Epi-F (RhoOGF, Hoechst, or Tf) or DIC microscopy. Bar = 10 µm.
served in the cytoplasm and the nucleus of EIPA-treated cells. Control experiments using transferrin Alexa Fluor 488 conjugate revealed a marked effect on inhibiting the entry of transferrin in cells treated with clathrin-related inhibitors (i.e., MDC and MβCD) but not EIPA.

**RhoOGF uptake is impaired by clathrin siRNA.** To further examine dependence of RhoOGF on a clathrin-mediated pathway, experiments with clathrin-targeted siRNA were conducted (Fig. 9). Clathrin expression was downregulated by 50% compared with that of scrambled siRNA-transfected cells (Fig. 9, top). Study of RhoOGF uptake with epifluorescent microscopy showed that clathrin depletion markedly diminished the entry of RhoOGF (Fig. 9, bottom).

**Dependence of RhoOGF on clathrin is consistent in human and animal cells.** Treatment of SKOV-3 and hMSC cells with clathrin siRNA significantly diminished the uptake of RhoOGF into these cells (Fig. 10).

**Clathrin is important for the inhibitory action of OGF on DNA synthesis.** To study whether clathrin is important to the function of the OGF-OGFr axis as an inhibitory pathway of cell proliferation, we examined DNA synthesis after transfection with clathrin siRNA (Fig. 11). Cultures transfected with clathrin siRNA had a 20% increase in cells incorporating BrdU compared with cells transfected with scrambled siRNA.

**Entry of OGF into cells is not related to endosomes or the Golgi apparatus.** To investigate the pathway of clathrin vesicles containing OGF, the relationship of OGF uptake to endosomes and the Golgi apparatus was examined. Neither endosomes nor the Golgi apparatus were observed to be colocalized with RhoOGF at a time when RhoOGF was in the cytoplasm only (15 min) or in both the cytoplasm and nucleus (60 and 120 min) (Fig. 12).

**DISCUSSION**

This is the first study to determine the pathway of OGF in live cells. Using RhoOGF, data mapping the temporal and spatial course of distribution of the entry of OGF into cells presented a number of lines of evidence to support the conclusion that OGF enters the cells by a process of active transport. First, when COS-7 cells were incubated with RhoOGF at 4°C, RhoOGF was not internalized into cells in a manner comparable to that at 37°C. These results indicate that the uptake of OGF into cells is an energy-dependent process that involves active transport. Second, when COS-7 cells were incubated with RhoOGF in the presence of OGF or the opioid antagonist NTX at 100 times the concentration of RhoOGF, RhoOGF was markedly diminished in uptake. However, other opioid peptides that recognized μ ([D-Ala², MePhe⁴, Glyol⁵]-enkephalin), δ ([D-Pen²,⁵]-enkephalin), and κ (U69,953 opioid receptor) did not attenuate RhoOGF uptake. Although concentrations of 1,000 times more of these opioid peptides as competitors would be even more assuring, these data suggest that RhoOGF entry into cells is acting as a saturable transport system. Third, COS-7 cells incubated with RhoOGF for as little as 15 min displayed rhodamine-labeled peptide in the cytoplasm, and within 30 min RhoOGF was detected in both the nuclear and cytoplasmic compartments. The subcellular location of RhoOGF was confirmed by deconvolution microscopy. These data demonstrate the rapid entrance of this compound into cells. Fourth, RhoOGF remained in cells for as long as 5 h, suggesting that OGF entry is acting as a saturable transport system.

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**Fig. 9.** RhoOGF uptake is impaired by clathrin siRNA. We seeded 2×10⁵ cells per well in 35-mm glass-bottom culture dishes containing 1 ml of media without antibiotics. Cells were transfected with 20 nM clathrin (Clath) siRNA or scrambled (Scram) siRNA. Seventy-two hours after transfection, cells were used for Western blot analysis (top) or RhoOGF uptake studies (bottom). Bar = 10 μm.
that the temporal course of this opioid peptide was of adequate duration to bind to the receptor and translocate into the nucleus. Fifth, two different cell types representative of a major human cancer (i.e., ovarian) and a human stem cell of mesenchymal origin, had an identical spatial and temporal distribution of RhoOGF. These results imply that active transport of OGF is a ubiquitous feature in human and animal cells, with no distinction between normal and malignant cell lines. Sixth, the pathway of OGF entry into cells (i.e., hMSC and OVCAR-3) with classical opioid receptors was similar to that of the COS-7 cells that do not have classical opioid receptors but do have OGFr (30). This paradigm of using COS-7 cells eliminated any confusion of interpreting the trafficking of OGF to multiple opioid receptors and demonstrated that RhoOGF entering cells is not dependent on pathways involving classic opioid receptors. Seventh, the uptake of RhoOGF was not due to the rhodamine moiety alone as rhodamine-labeled dextran did not enter cells and free fluorescein did not enter the nucleus. Therefore, in this report we make the

Fig. 10. RhoOGF uptake is dependent on Clath, a ubiquitous feature in human and animal cells. hMSCs and SKOV-3 cells were transfected with 20 nM Clath siRNA or Scram siRNA. Seventy-two hours after transfection, cells were used in RhoOGF uptake studies. Cells were stained with Hoechst and analyzed by Epi-F (RhoOGF or Hoechst) or DIC microscopy. Bar = 10 μm.

Fig. 11. Clath is important for OGF inhibitory function. COS-7 cells growing on 22-mm coverslips were transfected with Clath siRNA or Scram siRNA for 48 h. OGF was added to the cultures from 48 to 72 h. Fresh complete DMEM medium with 30 μM BrdU was added to each culture for an additional 3 h. Bars (means ± SE) represent the percentage of COS-7 cells that incorporated BrdU. *Data was statistically different from the control group at P < 0.05.
A seminal observation that OGF requires active transport to gain access to cells.

In a second series of experiments to determine the mechanism of OGF entry into cells, the data revealed a dependence on clathrin-mediated endocytosis. Evidence for this conclusion includes 1) a marked diminishment in RhoOGF uptake when cells were subjected to the clathrin-mediated endocytosis inhibitor MDC or MβCD (inhibits pathways dependent on caveolae/lipid rafts, as well as clathrin-mediated endocytosis) but not EIPA (an inhibitor of macropinocytosis) (7); 2) knockdown of clathrin by siRNA technology eliminated the entry of RhoOGF into animal and human cells; and 3) cells subjected to clathrin siRNA had a significant increase in BrdU labeling index because the tonic inhibitory effects of OGF were attenuated and thereby allowed

Fig. 12. RhoOGF is not colocalized with endosomes or the Golgi apparatus. A: COS-7 cells were subjected to RhoOGF (red) and examined 15, 60, and 120 min later for early endosomes (green). B: COS-7 cells were treated with RhoOGF (red) for 15, 60, or 120 min, and stained with antibody to the Golgi apparatus (green). Bar = 10 μm.
cells to accelerate in proliferation. Since clathrin-mediated endocytosis is often associated with early endosomes for sorting of cargoes as the next processing step before either recycling or degradation (8–10, 12, 19), and even a “retrograde transport” to the Golgi network (12), we asked whether RhoOGF was colocalized with early endosomes or the Golgi apparatus. Observations made at 15, 60, or 120 min, corresponding to the time span in which we recorded RhoOGF first in the cytoplasm and then both the cytoplasm and the nucleus, RhoOGF was never observed to be colocalized with either organelle. This would suggest that OGF is taken up by a clathrin vesicle and, after uncoating (11), does not take the endosomal (or Golgi apparatus) route but rather diffuses to the outer nuclear envelope to bind with OGFr. Thus, clathrin-mediated endocytosis appears to be a critical factor in the function of the OGF-OGFr axis in maintaining and regulating cellular proliferative activity in human normal and cancer cells.

OGF has a molecular weight of 574, whereas the rhodamine derivative of OGF attached to the C5/C6 carbons used in this study (i.e., RhoOGF) has a molecular weight of 988. It may be envisioned that the physiochemical properties of the rhodamine moiety could be important in determining the validity of the probe in terms of functional significance. Given that RhoOGF is a bulky molecule compared with the parent compound, the question can be raised as to whether the RhoOGF in the present study interferes with ligand-induced conformational changes related to localization, pharmacological properties, and cell proliferation. Structural studies in this investigation did reveal that RhoOGF was detected in both the cytoplasmic and nuclear compartments as recorded previously using antibodies to OGF (6, 28, 29). Pharmacologically, cold OGF in a concentration of 100 times that of RhoOGF was found to displace the cellular intake of RhoOGF. Moreover, as predicted, the opioid antagonist NTX also competed with RhoOGF and decreased RhoOGF uptake. Finally, OGF is known to depress DNA synthesis by exaggerating OGF-OGFr interactions, and a comparison was made to the effects of RhoOGF on cell proliferation. The results show that 24 h after exposure to RhoOGF the cells were downregulated in DNA synthesis to a magnitude comparable to that with OGF. Thus, the use of RhoOGF in determining the mechanics and pathway into cells also included the maintenance of function of this opioid peptide insofar as repressing cell proliferative activities.

In our initial exploration of the use of RhoOGF to examine the pathway into cells, we found that a concentration of $10^{-5}$ M, but not $10^{-6}$ M or less, was necessary to visualize the location of this fluorescent probe. Because previous investigators have reported that OGF concentrations of 1,000-fold less can decrease cell proliferation, it was valuable to understand whether this dosage of $10^{-5}$ M RhoOGF was critical to visualization and/or function, or if lower concentrations of RhoOGF could not be recorded by microscopy were functional. Investigation of the effects of $10^{-6}$, $10^{-7}$, and $10^{-8}$ M RhoOGF on DNA synthesis revealed that all three concentrations decreased DNA synthesis and did so to a magnitude comparable to $10^{-5}$ M. Moreover, at the same dosage (i.e., $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ M), the effects of RhoOGF on DNA synthesis were equipotent to OGF. Therefore, the concentration of RhoOGF required for visualization of this fluorescent probe is crucial but independent of RhoOGF’s capacity to repress cell proliferation.

Given the present information as to the means of entry of OGF, combined with previous reports using OGFr-eGFP and confocal, epifluorescent, and immunoelectron microscopic investigations (3, 6, 28, 29), the pathways of both OGF and OGFr, along with their interactions, can now be further defined (Fig. 13). OGF enters the cell by an active, energy-dependent process using clathrin-mediated endocytosis. The clathrin vesicle containing the OGF is uncoated after entry into the cytoplasm, and presumably the OGF diffuses to the OGFr (which takes at least 5.5 h for transcription and translation).

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**Fig. 13.** Schematic of a hypothetical model of the entry and nucleocytoplasmic pathway of OGF including the cell cycle and down-regulation of cell proliferation. A: OGF enters by active transport by Clath-dependent uptake, binds to the OGF receptor (OGFr) at the outer nuclear envelope, courses in the paranuclear cytoplasm, and enters to nucleus by the NLS-karyopherin β1/Ran system. Kap, karyopherin; NLS, nuclear localization signal. B: subsequent events related to the effects of OGF-OGFr activation on the signaling pathways related to the cell cycle. Cdk 4/2, cyclin-dependent kinase 4/2; p16/p21 proteins 16 and 21; Rb, retinoblastoma; G1 to S, gap phase1 to synthesis.
located on the outer nuclear envelope. The OGF-OGFr complex courses in the paranuclear cytoplasm, and the temporal sequence requires at least 15–30 min from the time of OGF entry, binding to the receptor, and shutting in the paranuclear cytoplasm. OGFr contains nuclear localization signals that interact with karyopherin-β and with Ran, traffic through the nuclear pore. This process appears to take as little as 15–30 min. The elements in the nuclear pore complex that are required for passage of the OGF-OGFr complex into the nucleus need to be clarified, as does the interactions of peptide-receptor system in the nucleus. The target of the OGF-OGFr system is the upregulation of p16 and/or p21, which leads to a marked delay in the G1/S phase of the cell cycle.

**Perspectives and Significance**

These results reveal that the opioid peptide regulating cell proliferation, OGF, enters cells by a process of active transport dependent on clathrin-mediated endocytosis. Disruption of clathrin-mediated endocytosis results in the loss of this native biological pathway and an upregulation of DNA synthesis with increase in cell number.

Patients with advanced pancreatic cancer have been treated with exogenous OGF (16, 17), and the safety and efficacy of this compound have been established. Based on the importance of the OGF-OGFr system in homeostasis of cellular renewal and the modulation of disease processes as shown in animals and humans (2, 4–6, 13), understanding the pathway(s) of OGF-OGFr action is important in elucidating the etiology and pathogenesis of irregularities in this system, as well as taking advantage of the OGF-OGFr axis for therapy. The present data revealing that OGF requires a controlled entry into the cell, implies that dysfunction of this machinery is crucial for OGF action. Therefore, patients with abnormalities of the clathrin-mediated endocytosis pathway may not be candidates for treatment with OGF. Moreover, whether defects in clathrin are a primary or secondary factor in disease pathogenesis by disabling a critical inhibitory pathway (i.e., OGF-OGFr) regulating cell proliferation, needs to be clarified.

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**DISCLOSURES**

I. S. Zagon and P. J. McLaughlin have a patent on OGFr.

**REFERENCES**