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Effects of GP2 expression on secretion and endocytosis in pancreatic AR4-2J cells

Su Yu, Ying Hao, Anson W. Lowe*

Department of Medicine and the Digestive Disease Center, Stanford University, Stanford, CA, USA

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Abstract

GP2 is the major membrane protein present in secretory granules of the exocrine pancreas. GP2's function is unknown, but a role in digestive enzyme packaging or secretion from secretory granules has been proposed. In addition, GP2 has been proposed to influence endocytosis and membrane recycling following stimulated secretion. Adenovirus-mediated GP2 overexpression in the rat pancreatic cell line AR4-2J was used to study its impact on digestive enzyme secretion and membrane recycling. Immunoelectron microscopy showed that GP2 and amylase co-localized in secretory granules in infected AR4-2J cells. CCK-8 stimulation resulted in a fourfold increase in amylase secretion with or without GP2 expression. GP2 expression also did not influence endocytosis following CCK-8 stimulation. Thus, GP2 expression in AR4-2J cells does not affect amylase packaging in secretory granules or stimulated secretion. GP2 expression also does not influence membrane recycling in response to stimulated stimulation in AR4-2J cells.

Keywords: Pancreas; Exocrine; Membrane protein; Secretion; Secretory granule; Secretory pathway; Endocytosis; GP2

Acinar cells present in the exocrine pancreas are responsible for digestive enzyme secretion in response to secretagogue stimulation [1]. The cells form polarized epithelia that secrete digestive enzymes into the pancreatic duct. Digestive enzymes contained within large secretory granules in acinar cells are secreted following cholecystokinin or cholinergic stimulation.

GP2 is the major membrane protein present in pancreatic exocrine secretory granules, comprising 35–45% of the total membrane protein [2,3]. The protein is linked to the membrane via a glycosylphosphatidylinositol linkage, but is cleaved from the membrane and secreted into the pancreatic duct along with the granule contents [4–6]. Because of its association with the proteins ZG16p and syncollin, GP2 has been proposed to form a submembranous matrix that functions in secretory protein packaging or granule formation [7–9]. GP2 has been also found to associate with amylase and other secretory proteins in vitro, supporting a potential role in secretory protein packaging or sorting to the secretory granule [10–14].

AR4-2J cells were originally derived from a rat pancreatic acinar tumor following exposure to azaserine [15]. The cells display many characteristics consistent with pancreatic acinar cells, including the presence of secretory granules and the stimulated secretion of amylase in response to cholecystokinin or acetylcholine [16]. Thus, AR4-2J cells have been used as a model system for pancreatic exocrine secretion. Stimulated secretion in AR4-2J cells, however, is suboptimal when compared to normal acinar cells in vivo [17]. The majority of amylase in AR4-2J cells is constitutively secreted soon after synthesis rather than stored in secretory granules. Because GP2 is expressed at very low levels in AR4-2J cells, they provided a suitable model system to test whether the protein can influence protein packaging or stimulated secretion [18].

* Corresponding author. Fax: +1 650 723 5488.
E-mail address: lowe@stanford.edu (A.W. Lowe).

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In addition to a role in protein packaging and secretion, GP2 has also been implicated to influence plasma membrane recycling following secretion [19–22]. GP2 is normally cleaved from the membrane and secreted. Defects in GP2 cleavage from the membrane have been observed in CFTR mutant mice, which has been proposed to disrupt the membrane recycling that normally follows stimulated secretion. Therefore, GP2 overexpression in AR4-2J cells also provided an opportunity to study the protein's impact on membrane recycling.

Materials and methods

Construction of adenovirus expression vector. Adenovirus containing the rat GP2 cDNA (Adeno-rGP2) was constructed using a modified version of adenovirus 5 [23]. The rat GP2 cDNA in pBluescript II KS (−) vector was subcloned into adenovirus shuttle plasmid vector, pHMCMV5, using the restriction sites *Xba*I/*Apa*I. The resultant plasmid pHMCMV5-rGP2 was then digested with restriction enzyme I-*Ceu*I/PI-*Sce*I and subcloned into the adenovirus expression vector, pAdHM10-lacZ (provided by Dr. Mark Kay, Stanford University). Adeno-rGP2 and wild-type adenovirus (Adeno-WT) were propagated in HEK293 cells and purified from culture media with centrifugation at 151,194*g* (SW41Ti rotor, Beckman Instruments, Palo Alto, CA) over cesium chloride step gradients with densities of 1.40 and 1.25 g/ml at 15 °C for 1 h. The virus band was recovered at the interface between the cesium chloride layers and further purified over another cesium chloride density gradient of 1.34 g/ml for 16 h at 151,194*g*. The virus layer was combined with an equal volume of buffer (100 mM Tris–HCl, pH 7.4, 10 mM EDTA, 100 mM NaCl, 1 mM $MgCl_2$, and 50% glycerol). The virus titer was determined with a plaque assay using HEK293 cells and the following formula: particles/ml = $(OD_{260} \times 20)/(9.09 \times 10^{13})$.

Cell culture and viral infection. AR4-2J cells (American Tissue Type and Culture, Bethesda, MD) were grown in Dulbecco's Modification of Eagle's media (DMEM) with 4.5 g/L glucose and L-glutamine (Cellgro, USA) containing 100 U/ml each of penicillin and streptomycin, and 10% fetal bovine serum. AR4-2J cells were pre-incubated for 72 h before an experiment with 10 nM dexamethasone (Sigma, St. Louis, MO) to increase amylase levels and the number of secretory granules [24]. The cells were plated in a 12-well tissue culture plate at 4×10^5 cells/2.2 cm diameter well for the pulse-chase studies or on 12 mm coverslips in a 24-well plate at 6×10^4 cells/well for the immunofluorescence studies. Twenty-four hours after plating, the cells were infected with Adeno-rGP2 or Adeno-WT at a multiplicity of infection of 100.

Pulse-chase studies. Pulse-chase studies were used to study the storage and secretion of amylase in the AR4-2J cells. Thirty-six hours after infection, the cells were pre-incubated for 30 min in methionine and cysteine-free DMEM plus 10% dialyzed fetal bovine serum (Hyclone, Logan, UT). The media were then exchanged for 0.5 ml of methionine and cysteine-free DMEM supplemented with 125 μCi [S^{35}]methionine (ICN, Irvine, CA) for 15 min. Following removal of the labeling medium, the cells were chased for 5 h in normal DMEM (1.0 ml) supplemented with 20 μM each of non-radioactive methionine and cysteine. Following the chase period, the media were exchanged for normal DMEM (1.0 ml) containing 250 nM CCK-8, an octapeptide derived from cholecystokinin, for 30 min. The media were then collected from each well and the remaining cells were lysed in the well with 1.0 ml NDET buffer (1% NP40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris–HCl, pH 7.4) containing 1 mM of phenylmethylsulfonyl fluoride and 10 μg of soybean trypsin inhibitor. The cell lysates and media were centrifuged at 12,000*g* for 10 min at 4 °C to remove the nuclei and cellular debris. Sodium dodecyl sulfate was then added to the cell lysate to a final concentration of 0.3% (w/v) [25]. A concentrated detergent-containing buffer was added to the media collected from the chase and CCK-8 stimulation such that the buffer composition was equivalent to the cell lysate. Quantitative immunoprecipitations for amylase were performed using an aliquot of the media and cell lysates. The samples were incubated at 4 °C overnight with a rabbit anti-human α-amylase antibody (Sigma, St. Louis, MO) and immunoprecipitated as previously described [25]. Data derived from the pulse-chase study were imaged with a phosphoimager (Bio-Rad FX Pro, Richmond, CA) and analyzed with Quantity One software (Bio-Rad).

Immunofluorescence microscopy. AR4-2J cells infected with Adeno-rGP2 or wild-type adenovirus were examined 36 h after infection. The cells were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.05% Triton X-100 in PBS for 15 min, and finally blocked with 5% FCS-50 mM NH_4Cl/PBS for 30 min. The cells were then incubated with the anti-GP2 monoclonal antibody (4A9, 1:300 dilution) and the rabbit anti-human α-amylase antibody (1:300 dilution, Sigma) for 1 h. After washing, the bound antibodies were labeled with FITC-conjugated donkey anti-mouse IgG (1:100 dilution) and Texas Red-conjugated donkey anti-rabbit IgG (1:100 dilution) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The nuclei were visualized using DAPI (Pierce, Rockford, IL). The labeled cells were viewed with a confocal laser scanning microscope (MRC 1024ES, Bio-Rad).

Electron microscopy. Electron microscopy was performed on AR4-2J cells infected in parallel with those prepared for confocal immunofluorescence. Cells were washed with PBS (pH 7.4), fixed with 0.1 M sodium phosphate buffer (pH 7.2) containing 2% glutaraldehyde (60 min, 4 °C). Further processing of the fixed cells was performed as previously described [26]. The cells were examined and photographed using a Philips CM-12 transmission electron microscope.

Electron micrographs of 100 individual randomly picked cells were photographed for each experimental condition. Differences in granule number between experimental conditions were assessed using Student's *t* test.

Immunoelectron microscopy was performed using the anti-rat GP2 antibody at 1:50 dilution and the anti-amylase antibody at 1:100 dilution. For double-labeling studies, 5 nm gold-conjugated donkey anti-mouse antibody (BB International, UK) was used for GP2 labeling and 10 nm gold-conjugated donkey anti-rabbit antibody was used for amylase labeling.

Endocytosis assay. AR4-2J cells were grown on glass coverslips and infected with adenovirus as previously described. Thirty-six hours after the adenovirus infection, the cells were washed three times with PBS and incubated for 30 min with regular media containing 250 nM CCK-8 and 16 μM FM4-64 (Molecular Probes, Leiden, Netherlands) [27]. The cells were washed three times with cold PBS, fixed with 4% paraformaldehyde/PBS for 5 min, sealed with mounting medium (Vectashield, Vector Laboratories, Burlingame, CA), and viewed with an immunofluorescence microscope (Nikon Eclipse E600, Nikon Instruments, Japan). Infection efficiency was assessed with GP2 immunofluorescence.

Images were obtained using a fluorescence microscope with a 60× oil objective and 532–587 nm filters, which includes the optimal absorption wavelength of 559 nm for FM4-64. The data were quantified by measuring the intensity of total FM4-64 signal per cell (Openlab software, Improvision, Coventry, UK) followed by an analysis using Student's *t* test.

Results

GP2 expression in AR4-2J cells

Rat GP2 was successfully expressed in AR4-2J cells with the Adeno-rGP2 virus as determined by

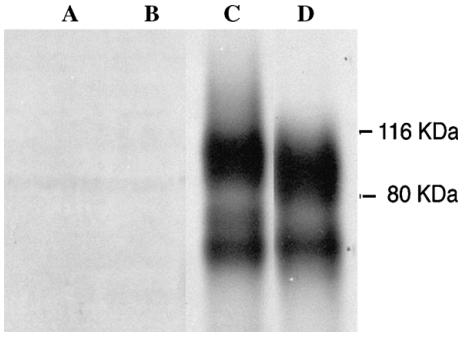

Fig. 1. Immunoprecipitation of rat GP2 from [S^{35}]methionine-labeled AR4-2J cells. Cells were labeled overnight with [S^{35}]methionine followed by immunoprecipitation from cell lysates with mouse anti-rat GP2 monoclonal antibody (4A9). Lane A, AR4-2J cells without infection; lane B, infection with Adeno-WT; lane C, infection with Adeno-rGP2; and lane D, MDCK cells that stably express rat GP2 as a positive control [25].

immunoprecipitation and SDS–PAGE analysis. Immunoprecipitation from [S^{35}]methionine-labeled AR4-2J cells produced the expected band at 95 kDa (Fig. 1). Two bands were seen representing mature and immature forms. The lower band (75 kDa) could be chased to the higher form with media without [S^{35}]methionine [25].

GP2 expression was also examined using immunofluorescence microscopy. In addition to intracellular staining, a significant fraction of the GP2 signal was located on the plasma membrane. No fluorescent signal was seen in uninfected AR4-2J cells or those infected with wild-type adenovirus (Fig. 2).

Amylase secretion in AR4-2J cells expressing rat GP2

The impact of GP2 expression on CCK-8 stimulated amylase secretion was assessed with pulse-chase studies using [S^{35}]methionine-labeled AR4-2J cells. The percentage of total cellular amylase secreted was assessed with and without CCK-8 stimulation in adenovirus-infected cells. CCK-8 stimulation resulted in a similar percentage of total cellular amylase secreted regardless of whether the AR4-2J cells expressed GP2 (Fig. 3).

Cellular amylase content was assessed with immunoblotting of cell homogenates. No difference in total cellular amylase content was seen whether or not the cells expressed GP2 (Fig. 3).

Secretory granule morphology

Immunoelectron microscopy studies were performed to determine whether GP2 was present in the secretory granules of cells infected with Adeno-rGP2. Double-immunogold labeling for amylase and GP2 showed that the two proteins were co-localized within secretory granules (Fig. 4D). GP2 labeling was detected

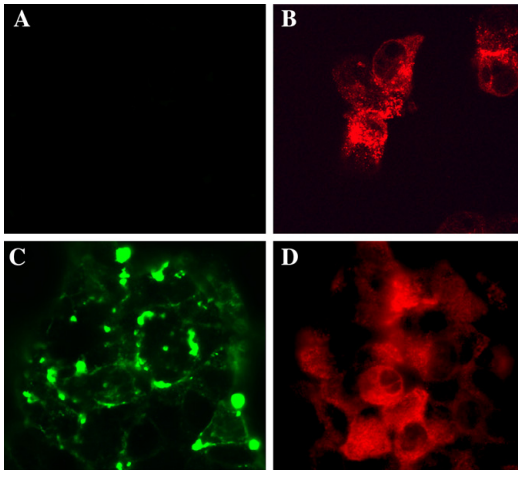

Fig. 2. Immunofluorescence of AR4-2J cells infected with Adeno-WT (A,B) or Adeno-rGP2 (C,D). The cells were double labeled for GP2 (A,C: FITC label) and amylase (B,D: Texas Red label). Magnification: 1000×.

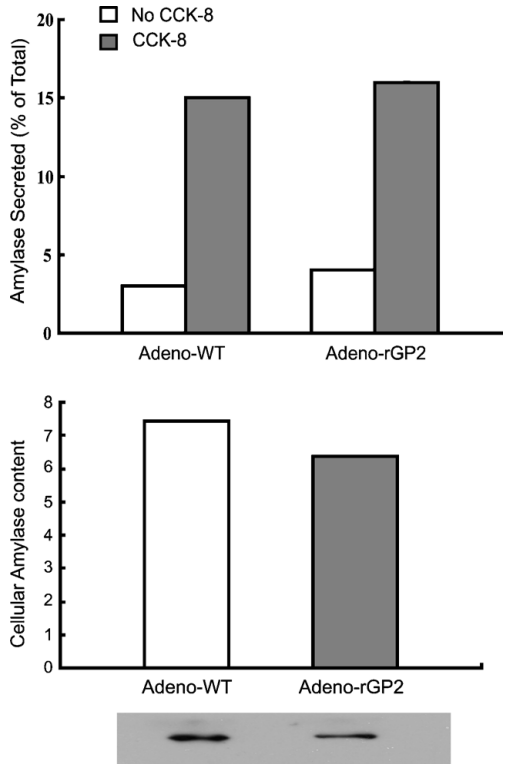

Fig. 3. (Top panel) Pulse-chase study for stimulated amylase secretion. AR4-2J cells were pulse labeled with [^{35}S]methionine for 15 min followed by a 5 h chase in non-radioactive media. The cells were then stimulated with CCK-8 (250 nM) for 30 min. Amylase was immunoprecipitated and quantified as described in Materials and methods. The data are presented as means ± 1SD (error bars). The data represent three independent experiments. (Bottom panel) Cellular amylase content was assessed with immunoblotting of total cell lysates collected from Adeno-WT- and Adeno-rGP2-infected cells. Immunoblotting was performed using the anti-human amylase antibody followed by densitometry analysis.

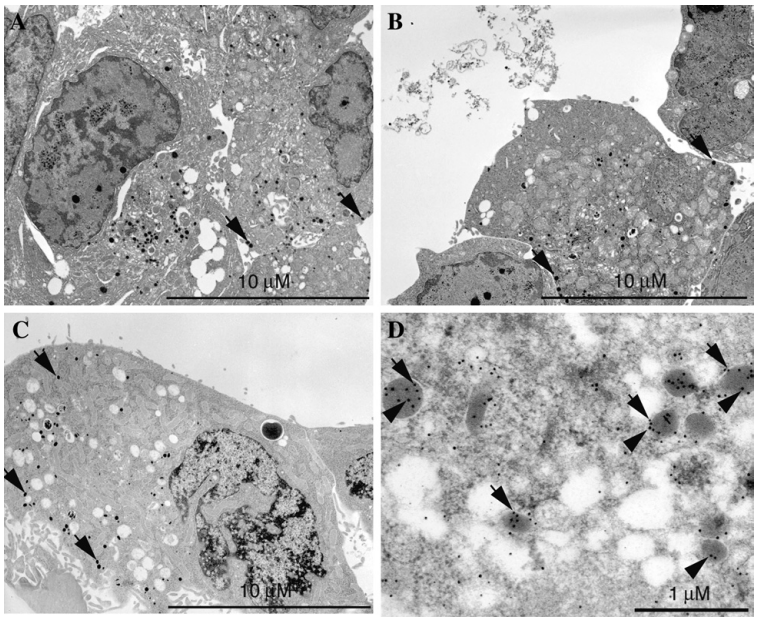

Fig. 4. Electron microscopy study of adenovirus-infected AR4-2J cells. (A) Non-infected AR4-2J cells; (B) Adeno-WT-infected cells; and (C) Adeno-rGP2-infected cells. Arrows in (A–C) identify zymogen granules. (D) Double staining with anti-GP2 (5 nm gold, arrow) and anti-amylase (10 nm gold, arrowhead) antibodies. (A–C) Magnification = 5000×; (D) 35,000×.

in secretory granules and the plasma membrane. In contrast, amylase was not detected on the plasma membrane.

Secretory granule number and morphology was also assessed with electron microscopy. The number of secretory granules in 100 Adeno-WT and Adeno-rGP2-infected AR4-2J cells was assessed. No significant difference in secretory granule numbers was seen between Adeno-WT and Adeno-rGP2-infected cells. Parallel immunofluorescence studies revealed that 92% of cells were infected and expressed GP2. A significant difference in secretory granule numbers, however, was found between non-infected and adenovirus-infected cells ($P < 0.01$) (Fig. 5). Adenovirus infection resulted in a sixfold decrease in secretory granule number.

GP2 overexpression and endocytosis

Endocytosis was examined using the fluorescent probe, FM4-64. FM4-64 uptake over 30 min was assessed in non-infected, Adeno-WT-infected, and Adeno-rGP2-infected cells with and without CCK-8 stimulation. No difference in FM4-64 uptake was observed in cells with CCK-8 stimulation (Fig. 6). GP2 expression in the infected cells was confirmed with immunofluorescence studies, which showed that 92% of all cells expressed GP2 (data not shown).

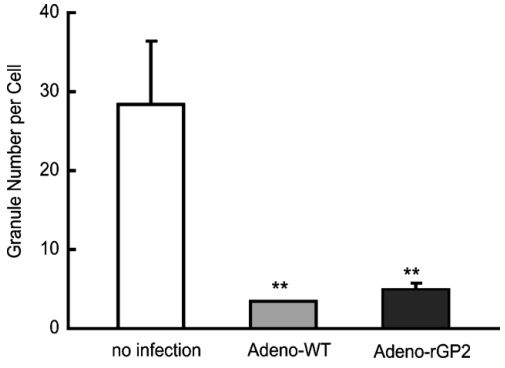

Fig. 5. Electron microscopy assessment of granule numbers. Granule numbers were counted and analyzed in 100 control cells, wild-type or GP2 adenovirus-infected cells. Data are presented as means ± 1SE of the mean. $^{**}P < 0.01$ for non-infected versus infected cell group.

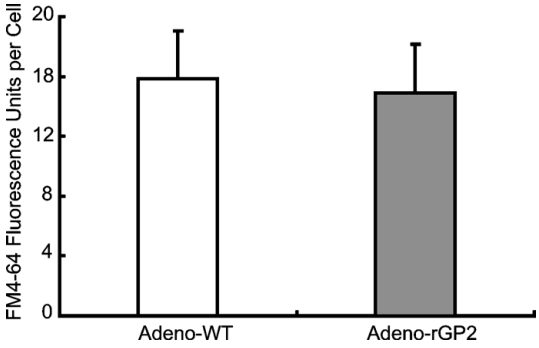

Fig. 6. Endocytotic activity measured by FM4-64 staining. Data represent the quantitative analysis of FM4-64 uptake between Adeno-WT and Adeno-rGP2-infected cells. Data are presented as means ± 1SD.

Discussion

Previous studies of GP2 in developing mice and AR4-2J cells concluded that the protein played no role in secretory granule biogenesis [18]. These studies demonstrated that the absence of GP2 in the developing mouse cells did not preclude secretory granule development. Recent studies, however, have suggested that GP2 associates with the secretory granule proteins ZG16p and syncollin to form a submembranous matrix that may participate in granule biogenesis or the packaging of secretory proteins [7–9].

The present study demonstrated that GP2 expression in AR4-2J cells does not affect the secretory process. Amylase was used as a marker of granule content because of its demonstrated interaction with GP2 in vitro, its abundance within the granule, and its known expression in AR4-2J cells. The pulse-chase studies showed that GP2 expression neither affects amylase packaging within secretory granules nor its secretion under the influence of CCK-8 stimulation. The results do not support a role for GP2 in amylase storage or stimulate secretion by secretory granules. Because AR4-2J cells were originally produced via chemical mutagenesis of rats, a definitive conclusion concerning GP2's role in secretion cannot be made because other proteins required for the secretory process may also be affected.

Endocytosis in AR4-2J cells was examined because of previous data suggesting that the presence of GP2 on the plasma membrane may influence membrane turnover following stimulated secretion [19,20,22,28,29]. GP2 is initially bound to the membrane as a glycosylphosphatidylinositol-linked protein. Upon secretion, GP2 is cleaved from the membrane and released into the pancreatic duct. GP2 cleavage from the membrane is inhibited by an acidic environment, which has been observed in GP2 expressing cell lines and in mice harboring mutations in the CFTR gene [22,25]. One hypothesis proposed suggests that the CFTR mutation results in impaired GP2 cleavage from the membrane [29]. Because GP2 displays homotypic interactions [11], endocytosis and membrane recycling may be affected when GP2 remains attached to the plasma membrane. Initial experiments with AR4-2J cells that express GP2 showed that much of the protein remained bound to the plasma membrane; presumably secondary to a compromised ability to cleave the protein. The increased presence of GP2 on the plasma membrane provided an opportunity to study its effects on endocytosis following stimulated secretion. Overexpression of GP2 did not affect endocytosis indicating that an increased fraction of plasma membrane bound protein does not necessarily lead to defects in membrane recycling.

Adenovirus was found to reduce the number of secretory granules detected with electron microscopy of infected AR4-2J cells. The impact of adenovirus infection on secretory granule number was independent of GP2 expression. Thus, the AR4-2J cells remain a viable system to study exocrine secretion using adenovirus-mediated gene expression, which carries importance in view of the paucity of pancreatic exocrine cell lines that are available and amenable to molecular manipulation.

Using the AR4-2J cells as a model system, this study does not support a role for GP2 in secretory granule biogenesis, protein packaging, or stimulated secretion.

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