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# Pancreatic Reg I Binds MKP-1 and Regulates Cyclin D in Pancreatic-Derived Cells

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***Background.*** **The pancreatic regenerating (reg I) gene and its protein product are derived from acinar cells and are mitogenic to β- and ductal cells. We studied the mechanism of this mitogenic response.**

***Materials and methods.*** **ARIP (rat ductal) and RIN 1046–38 (rat β-) cell lines were exposed to exogenous reg I in culture or transfected with a reg I expression vector. Mitogenesis was assessed by MTS assay (CellTiter 96; Promega, Inc., Madison, WI), and cellular mRNA was subjected to gene microarray analysis to determine signal transduction pathways. Yeast two-hybrid technology was then used to determine intracellular binding of reg I protein.**

***Results.*** **Cells exposed to exogenous reg I showed a mitogenic response; cells transfected with reg I expression vector showed inhibited growth. Microarray analysis of the former showed induction of cyclin pathways and mitogen-activated protein kinase phosphatase (MKP-1); cyclins were inhibited in the latter. Northern analysis confirmed gene induction of cyclin D1 and MKP-1; JNK was phosphorylated prior to expression of both. Yeast two-hybrid analysis confirmed a protein–protein interaction with MKP-1; this was confirmed by immunoprecipitation.**

***Conclusions.*** **Pancreatic-derived cells exposed to reg I grow by activation of signal transduction pathways involving the mitogen-activated protein kinase phosphatases and cyclins, with concomitant induction of MKP-1. However, high intracellular levels of reg I lead to decreased growth, likely via a binding to and inactivation of MKP-1. Inhibition of cell growth, and possible induction of apoptosis, may lead to differentiation of these cells to other cell types.** 

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***Key Words:*** **pancreatic reg I; pancreatic ductal cells; pancreatic β-cells; cyclin D1; MKP-1; EXTL3; mitogenesis.**

## INTRODUCTION

The regenerating (reg) family of genes is predominantly expressed in cells of the gastrointestinal tract [1–3]. Reg I is an acinar cell-derived product of the pancreas, is constitutively expressed and secreted from the acinar cells, and can be ectopically induced within regenerating islets. Reg I protein is an established mitogen to pancreatic β-, ductal, and mucosal cells of the gastrointestinal tract including the stomach and colon [2, 4, 5]. We have shown that purified recombinant reg I is bioactive as well [6] and is mitogenic to primary cultures of ductal cells [6].

The mechanism of reg I mitogenesis is likely paracrine. A transmembrane receptor has been identified that is homologous to the multiple exostoses-like gene family (EXTL3) [7]. EXT genes are mutated in bone disease, and the function of the EXT and EXTL proteins are in assembly of peptide-glycans, specifically, heparan sulfate [8, 9]. Specifically, they catalyze the polymerization of glycosaminoglycans into heparan sulfate; EXTL3 actually initializes the event [10]. However, EXT proteins have not been linked to cell growth, or cell cycle pathways, so precisely how reg I exerts its mitogenic effect is still unknown. While reg I does bind to the cell surface transmembrane EXTL3 protein, EXT gene products localize within the cell—specifically in the Golgi apparatus and endoplasmic reticulum [11]. Interestingly, Simmons *et al.* showed that EXT1 and EXT2 directly interact with intracellular proteins [12]. This raises the possibility that reg I might have a direct intracellular effect, by trafficking either into the cell by means of the receptor or after induction of its gene within the proliferating cells.



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To determine which intracellular signaling cascades are induced during mitogenesis by reg I, we used microarray analysis and found it to act through mitogen-activated protein kinase (MAPK) pathways in both ductal and β-cells. We also explored which proteins reg I associates with inside the cell using yeast two-hybrid technology and have determined that it actually binds to the MAPK phosphatase (MKP-1).

## MATERIALS AND METHODS

### Cell Proliferation and Gene Transcription

ARIP (rat ductal, American Type Culture Collection (ATCC, Rockville, MD)) or RIN 1046-38 (rat insulinoma) cells [6] were used. We defined 10 nM as the optimal dose for mitogenesis studies, since previous studies in our laboratory [6, 13] showed that reg I protein was mitogenic at doses ranging in concentration from 0.1 pM to 10 nM to both ARIP and RIN cells, but growth began to show inhibition at 100 nM.

To measure the effect of endogenously expressed reg I on these cell lines, a 540-bp cDNA sequence of the coding region of reg I [6] was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) between *Bam*HI and *Not*1 sites, under the control of the CMV promoter. Controls were cells transfected with pcDNA3 vector alone. Transfected cells were selected with 500 μg/mL G418 selection medium (Sigma). Reg I protein expression was verified through Western blotting with a monoclonal anti-reg I antibody 2B3-F12 developed in our laboratory. The antibody was created using reg I purified by serial ammonium sulfate precipitations from human pancreatic juice as previously described [13]. Monoclonal antibodies to reg I were raised according to established protocols. Briefly, female BALB/c mice were immunized with reg I in Freund's adjuvant (GIBCO Life Technologies, Inc., Rockville, MD). Hybridomas were produced using mouse myeloma cell line Sp2/0 (ATCC) and fused to mouse splenocytes using Hybrid MAX PEG Solution (Sigma). Supernatants of the clones were screened against purified rat and human reg I by enzyme-linked immunosorbent assay, and the positive clones were confirmed by Western blotting, using anti-mouse Ig–HRP (Amersham Biosciences, Piscataway, NJ) as a second antibody. Clone 2B3-F12 was chosen since it cross-reacted with both rat and human. Injection of the hybridoma into mice and harvesting of resultant ascites typically yielded a titer of 1:12,800 by enzyme-linked immunosorbent assay.

Normal and transfected ARIP ($100 \times 10^3$ cells/well) and RIN ($100 \times 10^3$ cells/well) were plated in 96-well plates, in Dulbecco's modified Eagle's medium with 10% fetal calf serum, penicillin, and streptomycin (GIBCO). After an overnight incubation, each well was washed three times with 1 mL phosphate-buffered saline (Sigma). Normal cells were inoculated with 10 nM recombinant reg I protein with 1% serum replacement medium (Sigma); pcDNA3-reg I transfected cells were compared to cells transfected with blank vector. Cells were incubated for 48 h and mitogenesis was assayed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)-tetrazoleum assay (CellTiter 96; Promega, Inc., Madison, WI) following the manufacturer's protocol. Absorbance was measured at 490 nm directly from 96-well assay plates with a microplate reader (Bio-Rad, Inc., Hercules, CA). Each experiment was done in triplicate and repeated at least twice.

For assessment of mitogen-activated kinase, ARIP ductal cells were inoculated with 10 nM recombinant reg I protein. A 24 h time course (0.5, 1, 4, 8, 24 h) of phosphorylation of the MAPKs, P44-42 (ERK1/2), P38, and SAPK/JNK was assessed on 10 μg of cellular protein by Western analysis using antibodies directed against the phosphorylated forms of the MAPKs (New England Biolabs, Ipswich, MA).

### Microarray Analysis of Reg I Gene Activation

One hundred micrograms of RNA was extracted from control and experimental ARIP and RIN cells with the RNeasy kit (Qiagen, Inc., Valencia, CA) and resuspended in diethylpyrocarbonate-treated water. Probes were prepared by annealing 17 μL/probe of resuspended RNA with oligo(dT) at 65°C and then labeled with either CY-3 (control) dUTP (green color) or CY-5 (experimental) dUTP (red color) fluorescent nucleotides with the Superscript-RT kit (GIBCO) during reverse transcription into cDNA. After RNase treatment, cDNA was concentrated (Amicon, Inc., Beverly, MA) and hybridized with mouse 9M gene chips obtained from, and following the protocol of, the Albert Einstein College of Medicine Microarray Core Facility. The gene chips were then scanned and analyzed with ScanAnalyze2 software (M.B. Eisen, Stanford University, Stanford, CA). The cutoff ratios for up-regulation and down-regulation were defined as 1.5 and 0.67, respectively. Each experiment was repeated three times.

### Isolation of Rat reg I Binding Proteins by the Yeast Two-Hybrid System

To identify genes encoding proteins that associate with reg I protein, we used the Hybrid ZAP-2.1 two-hybrid vector system (Stratagene, La Jolla, CA). The rat reg I coding sequence was cloned into pBD-GAL4 Cam (bait) phagemid vector, and a cDNA library of the target cell, ARIP, which was stimulated with recombinant reg I protein (10 nM for 2 d), was cloned into the pAD-GAL4-2.1 (target) phagemid vector using the manufacturer's guidelines.

The rat reg I coding sequence was established using PCR primers to directionally clone it into the pBD-GAL4 Cam (bait) vector using *Sal*I and *Pst*I at each end (reg I-*Sal*I primer was as follows: 5′ ACG C*GT CGA C*TC ATG ACT CGC AAC AAA TAT TTC 3′ (*Sal*I in italics), the sequence of reg I-*Pst*I primer was as follows: 5′ GGC A *CTG CA G* TCA GGC TTT GAA CTT GCA GAC 3′ (*Pst*I in italics). The recombinant pBD-GAL4-reg I plasmid was verified by DNA sequencing.

pBD-GAL4-reg I plasmid was transformed into freshly prepared yeast (YRG-2) competent cells, plated on SD agar plates without tryptophan, and incubated at 30°C for 2-4 d. Total RNA was extracted from the yeast colonies, and reg I mRNA was verified by RT-PCR. Reg I protein was confirmed by Western blotting with the anti-reg I monoclonal antibody described above.

Plasmid DNA from the pAD-GAL4-2.1 target library was transformed into the YRG-2 yeast containing pBD-GAL4-reg I. Selection was done on agar plates lacking histidine, leucine, and tryptophan. Colonies were transferred to nitrocellulose paper, permeabilized in liquid nitrogen, and assayed for expression of the *Lac*Z reporter gene by the detection of β-galactosidase.

Plasmid DNA was isolated from the His+*Lac*Z+ yeast colonies and transformed into XL1-Blue MRF′ competent cells. The target plasmid (pAD-GAL4-2.1) was selected by plating the transformant mixture on LB-Ampicillin agar plates. The target plasmid DNA was isolated using the B101 RPM Rapid Pure Miniprep Kit (Bio 101, La Jolla, CA). The size of the inserts in the target plasmids was analyzed using the Expand Long Template PCR System (Roche, Mannheim, Germany). The sequence of the 5′AD primer was as follows: 5′ AGG GAT GTT TAA TAC CAC TAC 3′; the sequence of the 3′AD primer was as follows: 5′ GCA CAG TTG AAG TGA ACT TGC 3′. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and elongation at 68°C for 6 min, followed by one cycle of final elongation at 68°C for 7 min. Resultant plasmid DNA was subjected to DNA sequencing, and the sequences were analyzed in GenBank using BLAST software.

### Immunoprecipitation/Western Blots

Whole cell lysates were prepared from ARIP and RIN cells, which were either normal or transfected with pcDNA3-reg I expression