Protein Synthesis and Degradation:

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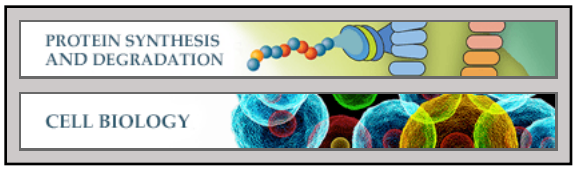

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Dynamic Modulation of Prohormone Convertase 2 (PC2)-mediated Precursor Processing by 7B2 Protein

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Michael Helwig[‡1], **Sang-Nam Lee**[§], **Jae Ryoung Hwang**[¶], **Akihiko Ozawa**[‡], **Juan F. Medrano**[‖], **and Iris Lindberg**[‡2]

From the [‡]*Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, the* [§]*Research Center for Natural Human Defense System, Yonsei University College of Medicine, Seoul 120-752, Korea, the* [¶]*Molecular Therapy Research Center, Sungkyunkwan University, Seoul 135-710, Korea, and the* [‖]*Department of Animal Science, University of California, Davis, California 95616-8521*

The small neuroendocrine protein 7B2 is required for the production of active prohormone convertase 2 (PC2), an enzyme involved in the synthesis of peptide hormones, such as glucagon and proopiomelanocortin-derived α-melanocyte-stimulating hormone. However, whether 7B2 can dynamically modulate peptide production through regulation of PC2 activity remains unclear. Infection of the pancreatic alpha cell line α-TC6 with 7B2-encoding adenovirus efficiently increased production of glucagon, whereas siRNA-mediated knockdown of 7B2 significantly decreased stored glucagon. Furthermore, rescue of 7B2 expression in primary pituitary cultures prepared from 7B2 null mice restored melanocyte-stimulating hormone production, substantiating the role of 7B2 as a regulatory factor in peptide biosynthesis. In anterior pituitary and pancreatic beta cell lines, however, overexpression of 7B2 affected neither production nor secretion of peptides despite increased release of active PC2. In direct contrast, 7B2 overexpression decreased the secretion and increased the activity of PC2 within α-TC6 cells; the increased intracellular concentration of active PC2 within these cells may therefore account for the enhanced production of glucagon. In line with these findings, we found elevated circulating glucagon levels in 7B2-overexpressing *cast/cast* mice *in vivo*. Surprisingly, when proopiomelanocortin and proglucagon were co-expressed in either pituitary or pancreatic alpha cell lines, proglucagon processing was preferentially decreased when 7B2 was knocked down. Taken together, these results suggest that proglucagon cleavage has a greater dependence on PC2 activity than other precursors and moreover that 7B2-dependent routing of PC2 to secretory granules is cell line-specific. The manipulation of 7B2 could therefore represent an effective way to selectively regulate synthesis of certain PC2-dependent peptides.

Prohormone convertase 2 (PC2)[3] belongs to the family of subtilisin-related proteolytic enzymes thought to mediate the majority of proteolytic cleavages of peptide hormone precursors (1–4). For example, during the multistep processing of the peptide precursor proopiomelanocortin (POMC), PC2 acts to cleave intact adrenocorticotropic hormone 1–39 (ACTH) into adrenocorticotropic hormone 1–14, the precursor to α-melanocyte-stimulating hormone (α-MSH). PC2 is also thought to be involved in the synthesis of β-endorphin and glucagon (4–6). The neuroendocrine protein 7B2, discovered in 1982 (7), facilitates the maturation of proPC2 and is absolutely required for expression of this convertase's enzymatic activity (8–12) (reviewed in Refs. 13 and 14). 7B2 accomplishes this by blocking the unproductive aggregation of the PC2 precursor into unactivatable aggregates (15). 7B2 itself is proteolytically cleaved from a larger form, 27-kDa 7B2, to its bioactive 21-kDa form by removal of a C-terminal domain. This removal is mediated by furin or a furin-like convertase within the *trans*-Golgi network (16). The amino-terminal 21-kDa domain is responsible for chaperoning the proPC2 maturation process (reviewed in Ref. 17).

Mice null for the 7B2 protein are unable to generate active pituitary prohormone convertase 2 (18). In these mice, the normal PC2-mediated process of internal cleavage of adrenocorticotropic hormone 1–39 does not occur in the intermediate lobe, despite the presence of ample quantities of proPC2 (18). Hypersecretion of adrenocorticotropic hormone 1–39 into the circulation ensues, which leads to adrenocortical stimulation, highly increased circulating corticosterone, and ultimately to numerous pathologies as a consequence of severe disturbance in the pituitary-adrenal axis (18–20). We have shown previously that administration of an adenoviral vector encoding 7B2 to 7B2 null mice results in a significant decrease in pituitary and circulating PC2 substrate adrenocorticotropic hormone 1–39 (21). Decreased adrenocorticotropic hormone is accompanied by decreased circulating corticosterone and increased circulating α-MSH. Oddly, however, overexpression of 7B2 in the adrenocorticotropic cell line AtT-20/PC2 does not increase cellular production of α-MSH (12).

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[2] To whom correspondence should be addressed: Dept. of Anatomy and Neurobiology, University of Maryland School of Medicine, Bldg. HSF II, Rm. S251, 20 Penn St., Baltimore, MD 21201. Tel.: 410-706-4778; Fax: 410-706-2512; E-mail: ilind001@umaryland.edu.

[3] The abbreviations used are: PC2, prohormone convertase 2; α-MSH, α-melanocyte-stimulating hormone; PC1/3, prohormone convertase 1/3; POMC, proopiomelanocortin; ANOVA, analysis of variance; RIA, radioimmunoassay.

Recently, Schmidt *et al.* (22) reported that various strains of mice expressing different levels of 7B2 exhibited circulating glucagon levels that correlated with cellular 7B2 but not with PC2 levels, strongly suggesting that 7B2 is rate-limiting for glucagon production. However, cellular production of glucagon was not systematically examined under conditions of 7B2 deficiency and surplus. In order to determine whether 7B2 levels can indeed dynamically modulate peptide production, we have investigated the role of over- and underexpression of 7B2 in various cell types and with different PC2-dependent precursors.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant Adenovirus—Recombinant adenoviruses encoding either 27-kDa 7B2 or β-galactosidase (as a control) were initially made by M. Castro and S. Windeatt and have been described previously by Sarac *et al.* (21). Viral stocks were assayed for the presence of replication-competent adenovirus using a replication competency assay by the supernatant rescue assay (23). The presence of endotoxin was also assayed (21). 911 cells were used for routine purification of adenoviruses. Double cesium chloride gradient centrifugation was performed on cell extracts using a Beckman SW41 rotor for 2 h (for the first centrifugation) and for 18 h (for the second centrifugation) at 24,000 × *g* at 4 °C. After isolation of the viral band, cesium chloride was removed using Sepharose CL-4B spin columns. The adenoviral particles were stored in 5% sucrose in virus storage buffer (150 mM NaCl, 20 mM Hepes, pH 7.8) at −70 °C (21). For some experiments, viruses were prepared by LSUHSC Vector Core Laboratories at similar titers and purity.

Cell Culture—Pituitary primary cell cultures were generated from 7B2 null mice sacrificed by decapitation. Pituitaries were removed from the sella turcica; two pituitaries per tube were placed in a 15-ml conical tube with Earle's medium containing 1% BSA (fatty acid-free) and 25 mM Hepes, pH 7.4. Collagenase solution, containing 4 mg/ml type IV collagenase (Sigma), 1 mg/ml hyaluronidase (Sigma), 0.01 mg/ml DNase I (Sigma), and 10 mg/ml fatty acid-free BSA (Roche Applied Science) in DMEM high glucose (4.5 g/liter D-glucose, catalogue no. 11995, Invitrogen), was added. Tissues were incubated for 5 min at 37 °C with shaking to dissociate the gland. Tissue fragments were collected by centrifugation for 5 min at 24,000 × *g* at room temperature; the supernatant was carefully removed and discarded. The tissues were then resuspended in 5 ml/tube of 3 mg/ml trypsin in DMEM. Resuspended tissues were incubated for 5 min at 37 °C and then centrifuged. The pellet was resuspended in 5 ml/tube of 1 mg/ml lima bean trypsin inhibitor (Sigma) in DMEM high glucose solution in order to neutralize trypsin. Cells were then washed with 3 ml of plating medium containing high glucose DMEM, 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA), and 0.5% gentamycin. Cells were plated into 24-well plates in 1.5 ml/well plating medium and incubated at 37 °C/5% CO_2. After 24 h of incubation, the medium was replaced with 2 ml of fresh plating medium. We used 2-day-old primary pituitary cell cultures for all experiments.

AtT-20 cells were obtained from the American Tissue Type Collection, whereas AtT-20/PC2 cells were obtained from Dr. Richard Mains (3). Both cell lines were cultured in DMEM (Invitrogen) containing 10% Nuserum (BD Biosciences), 2.5% FBS, and G418 (0.3 mg/ml) was added to AtT-20/PC2 cells to maintain PC2 expression. All cells were split into 6-well plates at 0.3×10^6 cells/well. After 24 h, one well of cells was counted to calculate the appropriate adenoviral vector titer. RinPE cells represent a stable rat insulinoma cell line originally derived from Rin5f and stably transfected with rat proenkephalin (24); these cells were maintained in DMEM containing 1 g/liter glucose (Invitrogen), 10% FBS, G418 (0.3 mg/ml); α-TC6 cells, a mouse alpha cell line, were grown in DMEM containing 1 g/liter D-glucose, 5% FBS, 15% horse serum (Irvine Scientific), and penicillin/streptomycin (100 units/μg/ml; Invitrogen).

Adenoviral Infection of Primary Cultures—Each adenoviral preparation was diluted to 5×10^6 plaque-forming units (pfu)/well for pituitary primary cell cultures at a multiplicity of infection of 1. Each well was washed twice with PBS and infected with diluted recombinant adenoviral vector. After incubation for 30 min at room temperature, 2 ml of the growth medium were added to each well, and cells were then incubated for 24 h at 37 °C/5% CO_2.

Adenoviral Infection of Cell Lines—To infect adenovirus into cells, AtT-20/PC2, α-TC6, and/or RinPE cells were split into 6-well plates at 10^6 cells/well and 0.3×10^6 cells/well, respectively. Replicate wells were counted again the following day for calculation of adenoviral multiplicity of infection. Cells were washed twice with PBS, and adenovirus was diluted to achieve a multiplicity of infection = 1 (for AtT-20/PC2 and α-TC6 cells) or 3 (for RinPE cells) in PBS in a final volume of 1 ml/well. The diluted adenovirus solution was added directly to cells in growth medium, and the plates were swirled to mix well. The plates were incubated for 30 min to permit adenoviral infection. Two ml of DMEM containing high glucose and 2% FBS for AtT-20/PC2 cells and DMEM containing low glucose and 2% FBS for RinPE cells were then added to each well. Adenovirus-infected cells were incubated for 36 h at 37 °C in a CO_2 incubator. The medium was then changed overnight to Opti-MEM (Invitrogen) containing 0.1 mg/ml aprotinin for PC2 assays; alternatively, cells were used for metabolic labeling with [^{35}S]Met/Cys (Amersham Biosciences). Transfection efficiency was tested in simultaneous experiments by visualizing transfected cells using a β-galactosidase-encoding control adenovirus at the same concentration.

RNAi Experiments—Three different specific sequences of stealth siRNA (Invitrogen) were designed for the murine 7B2 mRNA sequence (MSS237887, MSS237888, and MSS237889). Following assessment of individual knockdown efficiencies, the most effective siRNA, MSS237887, was deployed in all experiments described here. A control scrambled sequence was designed to have the same GC content (46-2000, Invitrogen). Transfection efficiency in α-TC6 cells was monitored and visualized using a scrambled siRNA sequence conjugated to fluorescein (N2100S, New England Biolabs (Ipswich, MA)). The total cell number was determined by counterstaining with 5 μg/ml Hoechst 33342 (Invitrogen) for 45 min and subsequent examination by fluorescence microscopy. AtT-20/PC2 and

α-TC6 cells grown in 6-well plates were transfected sequentially with a 100 nM concentration of the respective siRNA on the first day and 200 nM on the second day using 5 μl/well Lipofectamine 2000 (Invitrogen).

Metabolic Labeling and Immunoprecipitation—After a 24-h infection with adenoviruses encoding either 27-kDa 7B2 or β-galactosidase control adenovirus, AtT-20/PC2 and RinPE cells were labeled with [^{35}S]Met/Cys Promix for 20 min in DMEM Met/Cys-free medium (ICN) and chased for 2 h in DMEM high glucose medium and 2% fetal bovine serum at 37 °C. The chase medium and cell extracts were immunoprecipitated using either PC1/3 antiserum (2B6), PC2 antiserum (18B8), or 7B2 antiserum (13B6), as described previously (7). Triplicate samples (wells) of immunoprecipitated proteins were separated by SDS-PAGE and visualized using a Storm 840 PhosphorImager (Amersham Biosciences). The density of bands was determined using an Alpha Imager 3000 system (Alpha Innotech Corp., San Leandro, CA). The expression levels of 7B2, PC1/3, and PC2 were calculated as integrated density of bands (arbitrary units $\times 10^{-5}$) and converted to percentages of non-treated controls ($n = 3$/group; data not shown).

Labeled proenkephalin present in RinPE cells was immunoprecipitated using antiserum directed against its C-terminal heptapeptide (Tyr-Gly-Gly-Arg-Phe) and analyzed at different chase times following separation by high pressure gel permeation chromatography, as described previously (24). Online scintillation spectroscopy was used to follow labeled methionine present in the various cleavage products.

Pituitary primary cell cultures were washed with PBS after adenoviral infection for 24 h and then labeled with 0.5 mCi/ml ^{35}S-labeled methionine and cysteine (Met/Cys) Promix in methionine-free medium for 20 min and chased for 2 h in medium containing cold methionine. After adenoviral infection, AtT-20/PC2 cells were incubated for 36 h, washed with PBS, and then labeled as described above. Cell extracts and conditioned media were then immunoprecipitated using either PC2 or 7B2 antisera.

AtT-20 cells overexpressing PC2 were first transfected in 12-well plates with 250 μl of DMEM containing 250 pM 7B2 siRNA (MSS237887) or 5 nM dsRNA using Lipofectamine 2000 (Invitrogen) and then incubated for 24 h at 37 °C. The cells were further co-transfected with the same concentration of 7B2 siRNA (or control dsRNA) and 1 μg of plasmid encoding human preproglucagon (pTT-proglucagon, a kind gift of Five-Prime Therapeutics, Inc.). Accordingly, α-TC6 cells were co-transfected with 2 μg of plasmid encoding POMC. After 24 h of incubation, the transfected cells were labeled with 0.25 mCi of ^{35}S-labeled methionine and cysteine, Transmix (MP Biomedicals, Solon, OH) in RPMI-1680, methionine- and cysteine-free medium (Invitrogen) for 20 min and chased for appropriate time periods in Opti-MEM (Invitrogen) containing 0.1 mg/ml aprotinin. Labeled proteins were extracted with 1 M acetic acid and clarified by centrifugation. The cell extracts were lyophilized and reconstituted with immunoprecipitation buffer containing 100 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 0.5% Nonidet P-40. One-third of the reconstituted cell extract was reserved in order to perform radioimmunoassays to measure cellular α-MSH and glucagon. Immunoprecipitations of clarified extracts were performed using either LS-41 antiserum (for POMC and POMC-derived products (25)) or glucagon antiserum (Takara Bio, Inc., Otsu Shiga, Japan) and protein A beads (GE Healthcare). Immunoprecipitates were subjected to separation by high performance gel permeation chromatography, as described previously (24), and scintillation counting of fractions was performed using a Beckman C5580 scintillation counter (Beckman Coulter, Brea, CA).

Secretion Experiments and Radioimmunoassays—Twenty-four h after infection, wells were washed twice with Opti-MEM and incubated with 1 ml of Opti-MEM containing 0.1 mg/ml aprotinin and BSA (5 μg/ml) for 1 h at 37 °C. The medium was replaced with 1 ml of Opti-MEM containing 0.1 mg/ml aprotinin and BSA (5 μg/ml) for 2 h at 37 °C and then collected. For stimulation experiments, 1 ml of Opti-MEM containing 100 μg/ml aprotinin, BSA (5 μg/ml), and 5 mM $BaCl_2$ was added to each well and then incubated for 1 h at 37 °C; in some experiments, RPMI 1640 was used instead of Opti-MEM containing 0.1 mg/ml aprotinin. After the conditioned media were collected, cells were homogenized immediately in 1 ml of acid mix (5 N acetic acid and 2 mg/ml BSA). The homogenized cells were frozen and then thawed and centrifuged. The supernatant was lyophilized and resuspended in 500 μl of RIA buffer (50 mM sodium phosphate, pH 7.6, 0.1% Triton X-100, and 0.02% sodium azide).

For α-MSH assays, 5 μl of cell extracts, 50 μl (for basal secretion) or 25 μl (stimulated secretion) of medium samples from pituitary primary cell cultures were subjected to assay in duplicate using the α-MSH RIA kit (Phoenix Pharmaceuticals, Burlingame, CA). This anti-α-MSH-antiserum does not recognize ACTH. For α-MSH assays using cell extracts and medium samples obtained from α-TC6 and AtT-20/PC2 cells, the polyclonal anti-α-MSH antiserum was commercially purchased from Chemicon (Temecula, CA). Two μm of cell extracts, 25 μl (for basal secretion) or 5 μl (stimulated secretion) of medium samples were subjected to assay in duplicate. ^{125}I-Labeled α-MSH was prepared by the chloramine-T method originally described by Hunter and Greenwood (26). RIA was carried out according to protocols described previously (27). Samples were incubated with 10,000 cpm of iodinated peptide and the appropriate dilution of rabbit antiserum in a final volume of 300 μl at 4 °C overnight. To separate the antibody-bound labeled peptide from the unbound labeled peptide, 1 ml of 25% polyethylene glycol and 100 μl of 7.5% carrier bovine γ-globulin (in PBS) were added. The samples were vortexed, kept on ice for 30 min, and then centrifuged for 20 min at 3,000 × *g* at 4 °C using a Sorvall RT6000B refrigerated centrifuge. The supernatant was aspirated, and the radioactivity in the pellets was determined.

For ACTH assays, 5 μl of a 1:20 dilution of cell extracts, 5 μl (for basal secretion) or 2 μl (stimulated secretion) of medium samples from pituitary primary cell cultures were subjected to assay in duplicate using the two-site Nichols human ACTH 1–39 assay kit (Nichols Institute, San Juan Capistrano, CA). For AtT-20/PC2 cells, 2 μl of cell extracts, 25 μl (for basal medium samples) or 5 μl (stimulated medium samples) of medium samples were assayed. The ^{125}I-ACTH antibody used in this kit is directed to both N-terminal and C-terminal regions of intact

ACTH molecule and does not recognize ACTH cleavage products. Radioactivity was determined using a Wallac 1470 Wizard γ-counter (PerkinElmer Life Sciences).

For glucagon assays, 48 h after transfection with the respective siRNA, each well was washed twice with PBS (pH 7.4). The PBS was replaced with 1 ml of 0.1 M HCl, and cells were collected by scraping, frozen on dry ice, thawed, and centrifuged. The supernatant was lyophilized and resuspended in 500 μl of RIA buffer (50 mM sodium phosphate, pH 7.6, 0.1% Triton X-100, and 0.02% sodium azide) and centrifuged again. Ten μl of clarified α-TC6 cell extract (7B2 RNAi-treated and scrambled controls) were subjected to assay in duplicate using a commercially available glucagon RIA kit (GL-32K, EMD Millipore, Billerica, MA). The anti-glucagon antiserum utilized in this kit recognizes processed glucagon but not its precursor proglucagon or oxyntomodulin. Samples were assayed using the RIA protocol according to the manufacturer's instructions. Radioactivity was determined using a Wallac 1470 Wizard γ-counter.

Enzyme Assays—After adenoviral infection of cell cultures for 24 h, the medium was changed to 1 ml of Opti-MEM containing 0.1 mg/ml aprotinin, and cells were allowed to secrete for 18 h. The assay for PC2 in this conditioned medium was carried out in 96-well polypropylene microtiter plates using 25 μl of each conditioned medium sample in a total volume of 50 μl, containing 200 μM fluorogenic substrate, Pyr-Arg-Thr-Lys-Arg-methylcoumarinamide (Peptides International, Louisville, KY), and 100 mM sodium acetate, pH 5.0, 5 mM $CaCl_2$, and 0.1% Brij 35, in the presence of a protease inhibitor mixture composed of 1 μM pepstatin, 0.28 mM tosylphenylalanyl chloromethyl ketone, 1 μM *trans*-epoxysuccinic acid (E-64), and 0.14 mM tosyllysyl chloromethyl ketone. The rate of released 7-amino-4-methylcoumarin was measured with a Fluoroscan Ascent fluorometer (MTX Lab Systems, Inc., Vienna, VA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for 1 h at 37 °C. Inhibition by the 7B2 C-terminal peptide was used to assess specificity as described previously (28).

Animals—Intercrosses (B62D-3 F2) of the subcongenic inbred strain (B62D-3, B6.CAST-(D2Mit329-D2Mit457)) were maintained at the University of California (Davis, CA). 7B2 is differentially expressed in these congenic mice, with relatively low mRNA and protein levels in the *b6/b6* genotype (considered as normal expressers), whereas *cast/cast* mice are high 7B2 expressers with levels increased 2.5-fold over normal (29). Expression of 7B2 in heterozygous *b6/cast* mice is intermediate. Samples of blood plasma were taken from male B62D-3 F2 mice, and glucagon levels in 100 μl of blood plasma were quantified by RIA, as described above (*b6/b6*, $n = 8$; *b6/cast*, $n = 3$; *cast/cast*, $n = 8$). Blood glucose levels were measured in duplicate using the same blood plasma samples using a handheld glucose meter (Freestyle mini, Abbott (Abbott Park, IL)). All animal protocols were managed according to the guidelines of the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Statistical Analysis—Data were analyzed with one- or two-way ANOVA followed by the Student-Newman-Keuls multiple comparison test, as appropriate, using a statistical software package (SigmaStat, Systat Software, Inc. (San Jose, CA)). Where data failed equal variance or normality tests, they were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. A probability value of $p < 0.05$ was considered as statistically significant.

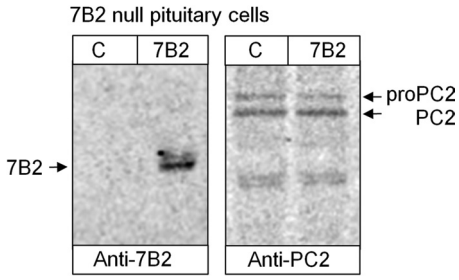


FIGURE 1. **Infection of primary cultures of 7B2 null pituitaries with 7B2-encoding adenovirus results in efficient expression of 7B2 but does not affect proPC2 processing.** Primary pituitary cell cultures derived from 7B2 null mice were infected with either (control) β-galactosidase- or 7B2-encoding adenovirus at 0.5×10^6 pfu/well in 12-well plates and subjected to metabolic labeling experiments as described under "Experimental Procedures." Cells were extracted with boiling immunoprecipitation buffer. Cell extracts were immunoprecipitated using antisera against 7B2 (*left*) or PC2 (*right*) under denaturing conditions. Although adenoviral transfection of pituitary cells with a 7B2-expressing vector induced an efficient expression of 7B2, no effect on the conversion of proPC2 to PC2 was detected in these cells. *C*, control; *7B2-AV*, 7B2 adenovirus.

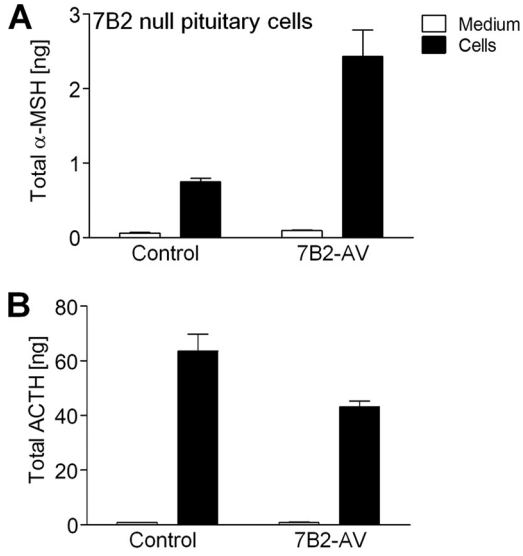


FIGURE 2. **Infection of primary cultures of 7B2 null pituitaries by 7B2-encoding adenovirus increases stored α-MSH levels and decreases ACTH levels.** Primary pituitary cell cultures derived from pituitaries of 7B2 nulls were infected with β-galactosidase (*Control*)- and 7B2-expressing adenoviral vectors with 0.5×10^6 pfu/well in 12-well plates for 24 h. Acid extracts were lyophilized and then resuspended in RIA buffer and subjected to radioimmunoassay for determination of α-MSH (*A*) or ACTH levels (*B*). Expression of 7B2 was accompanied by an increase in cellular α-MSH and a drop in ACTH levels. Levels of secreted α-MSH and ACTH in the medium were very low, and no differences between 7B2 expressing and non-expressing cells were detectable. *7B2-AV*, 7B2 adenovirus. Data represent $n = 3$ wells/group, mean ± S.D. (*error bars*).

RESULTS

7B2 Adenoviral Infection Increases ACTH Processing to α-MSH in Primary Pituitary Cultures Prepared from 7B2 Null Mice—Primary cell cultures derived from pituitaries of 7B2 null mice were infected with either β-galactosidase-encoding control virus or 27-kDa 7B2-encoding virus, respectively, labeled with radioactive methionine/cysteine; cell extracts were immunoprecipitated using antisera against PC2 and 7B2. 7B2 expres-

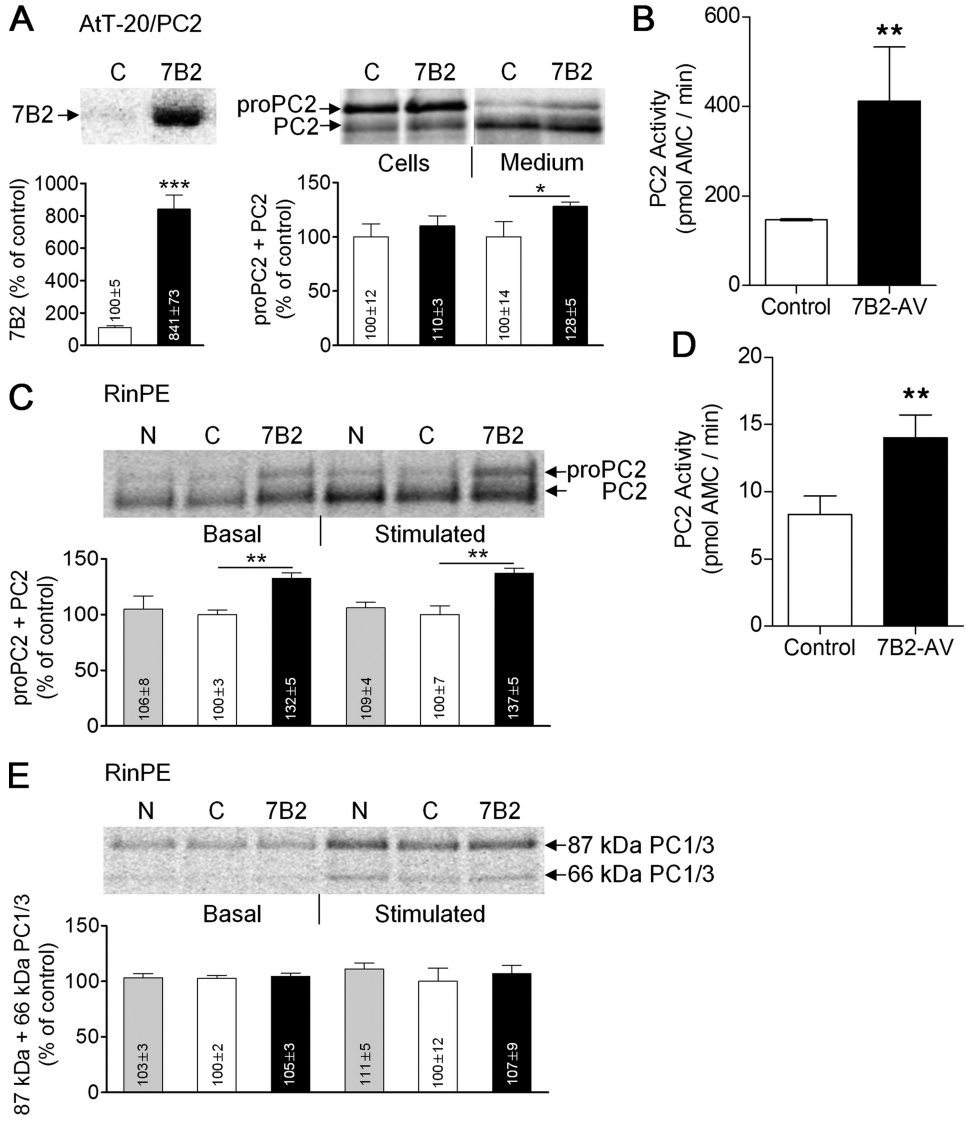


FIGURE 3. **Infection of different endocrine cell lines with 7B2-encoding adenovirus selectively increases the secretion and activity of proPC2/PC2, but not PC1/3, in RinPE cells.** AtT-20/PC2 cells and RinPE cells were infected with 10 pfu/cell and 3 pfu/cell of either control or 7B2-encoding adenovirus, respectively. Twenty-four h after infection, cells and media were subjected to metabolic labeling and enzyme activity experiments as described under "Experimental Procedures." *A*, cell extracts of AtT-20/PC2 cells were immunoprecipitated using antisera against either 7B2 (*left*) and PC2 (*right*) under denaturing conditions. *Graphs below* the representative blots show quantification of protein levels after normalization to protein content in control groups ($n = 3$). Overexpression of 7B2 in AtT-20/PC2 cells induced an increase of proPC2 and PC2 in the medium, but no difference within cells was observed. *B*, the increased secretion of PC2 was accompanied by an increase in enzyme activity in the medium as determined by PC2 enzyme assay of conditioned medium. *C–E*, metabolic labeling and immunoprecipitation demonstrates enhanced secretion of PC2 and proPC2 (*C*) but not PC1/3 (*E*) in the medium of 7B2-overexpressing RinPE cells under basal and stimulated secretory conditions. The enhanced PC2 release in these cells was also reflected by an increase in PC2 activity in the medium (*D*). *7B2-AV*, 7B2 adenovirus; *C*, control adenovirus; *N*, normal without adenovirus. Data represent $n = 3$ wells/group, mean ± S.D. (*error bars*); one-way ANOVA; **, $p > 0.01$.

sion was robust in the primary cell cultures transfected with 7B2-encoding virus (Fig. 1, *left*). Despite the fact that stable overexpression of 7B2 enhanced proPC2 processing to 68-kDa PC2 in AtT-20 cells (12), cleavage of proPC2 still occurred efficiently in primary cell cultures in the absence of 7B2, possibly by other convertases (30) (Fig. 1, *right*).

7B2 overexpression resulted in increased cellular levels of α-MSH (Fig. 2*A*) and decreased levels of ACTH (Fig. 2*B*), whereas concentrations of both peptides were very low in the medium. These results indicate that overexpression of 7B2 increases POMC processing in primary cultures of 7B2 null pituitary, similar to its effect in the intact animal (18).

7B2 Adenoviral Infection Enhances proPC2 Secretion and Increases Secretion of Active PC2 in AtT-20/PC2 and RinPE Cells—In order to determine whether 7B2 overexpression can affect POMC processing and secretion in neuroendocrine cell lines, we infected 7B2-encoding adenovirus into AtT-20/PC2 cells (these cells represent a PC2-transfected AtT-20 cell line known to process the endogenous substrate, POMC, at PC2 cleavage sites (31)) and RinPE cells (a rat insulinoma cell line stably overexpressing rat proenkephalin (24)). ^{35}S labeling of AtT-20/PC2 cells showed that 7B2 was indeed well expressed in these cells (Fig. 3*A*, *left*). Adenoviral 7B2 overexpression did not affect proPC2 processing into PC2 in cells infected with 7B2

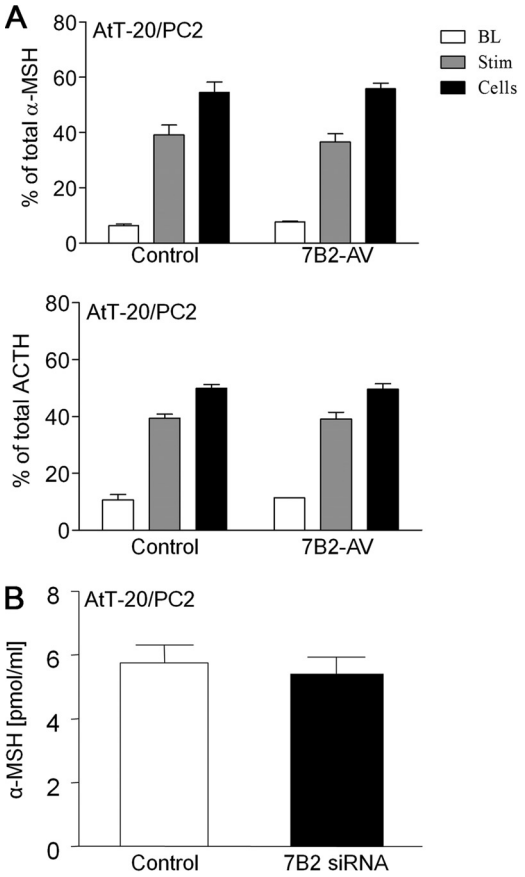


FIGURE 4. **Manipulation of 7B2 levels in AtT-20/PC2 cells by adenoviral infection or antisense does not alter POMC processing or enhance secretion of α-MSH or ACTH.** *A*, neither secreted nor intracellular levels of α-MSH or ACTH change following 7B2 overexpression. AtT-20/PC2 cells were infected for 24 h with control adenovirus encoding either β-galactosidase or 7B2. Following a 40-min basal collection period, cells were incubated in medium containing 5 mM $BaCl_2$ for 40 min to assess stimulated secretion. Cell extracts and medium samples were assayed for α-MSH and ACTH. Data are presented as a percentage of total peptide ($n = 3$ wells/group). *BL*, peptide released under basal conditions; *Stim*, peptide released during $BaCl_2$ stimulation; *Cells*, peptide present in cells. *B*, RNAi-mediated knockdown of 7B2 does not alter intracellular content of α-MSH. AtT-20/PC2 cells were transfected for 48 h with either scrambled control siRNA or 7B2 siRNA, and cell extracts were assayed for α-MSH. Data represent $n = 3$ wells/group, mean ± S.D. (*error bars*).

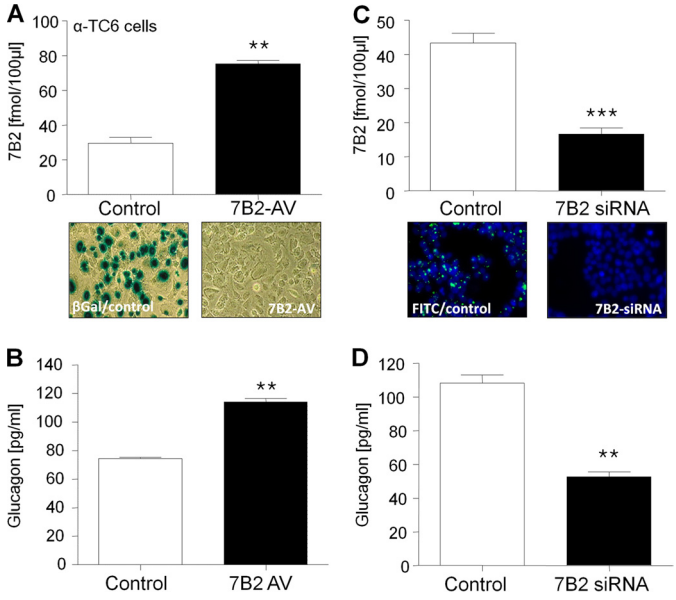


FIGURE 5. **7B2 adenoviral infection of α-TC6 cells enhances the production of cellular glucagon, whereas transfection with 7B2 antisense siRNA lowers glucagon levels.** α-TC6 cells were infected for 24 h with control adenovirus encoding either β-galactosidase (*βGal/control*) or 7B2 (*7B2-AV*). 7B2 overexpression by adenoviral infection in α-TC6 cells increases 7B2 levels (*A*). 7B2-AV transfection efficiency was determined using a β-Gal-encoding adenovirus. Representative photomicrographs show β-Gal/control and 7B2-transfected α-TC6 cells. The adenovirus-induced overexpression of 7B2 is accompanied by increased cellular glucagon levels (*B*). RNAi-mediated knockdown of 7B2 was efficiently induced using 7B2-specific siRNA (*C*). Representative photomicrographs show FITC-labeled scrambled siRNA control (FITC/control) and 7B2-siRNA transfected α-TC6 cells. Decrease of intracellular 7B2 is associated with a significant drop of intracellular glucagon levels (*D*). Data represent $n = 3$ wells/group, mean ± S.D. (*error bars*); one-way ANOVA; **, $p > 0.01$; ***, $p > 0.001$.

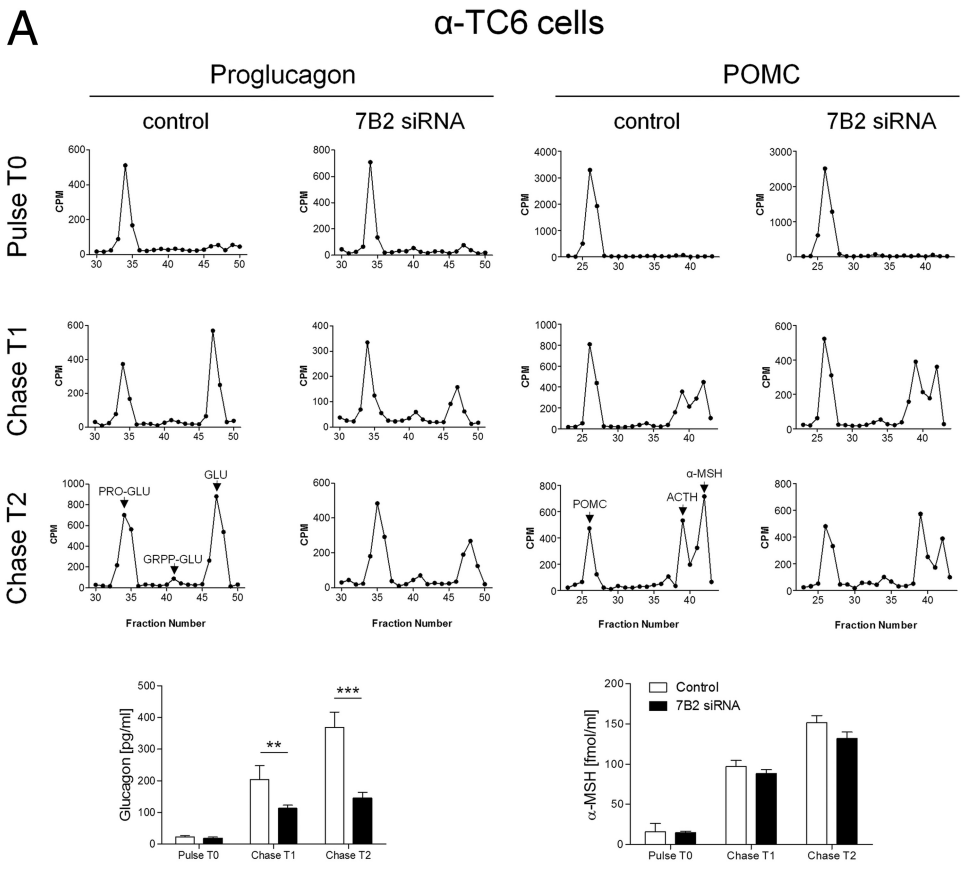
adenovirus (Fig. 3*A*, *right*). Secretion of proPC2 and PC2 from AtT-20/PC2 cells was, however, slightly increased after adenoviral transfection with 7B2. Fig. 3*B* shows that the 7B2-enhanced release of proPC2/PC2 from AtT-20/PC2 cells is reflected in a large increase in the release of PC2 activity. In RinPE cells, adenoviral 7B2 overexpression also increased secretion of radiolabeled proPC2 and PC2 into the medium; this increase in secretion was observed when secretion was either non-induced or stimulated by $BaCl_2$ (Fig. 3*C*). The increase of PC2 secretion in RIN-PE cells was accompanied by an increase of PC2 activity in the medium (Fig. 3*D*). In contrast to the observed changes in proPC2 and PC2 secretion, the secretion of radiolabeled PC1/3 was not altered by the introduction of 7B2 adenovirus into RinPE cells (Fig. 3*E*).

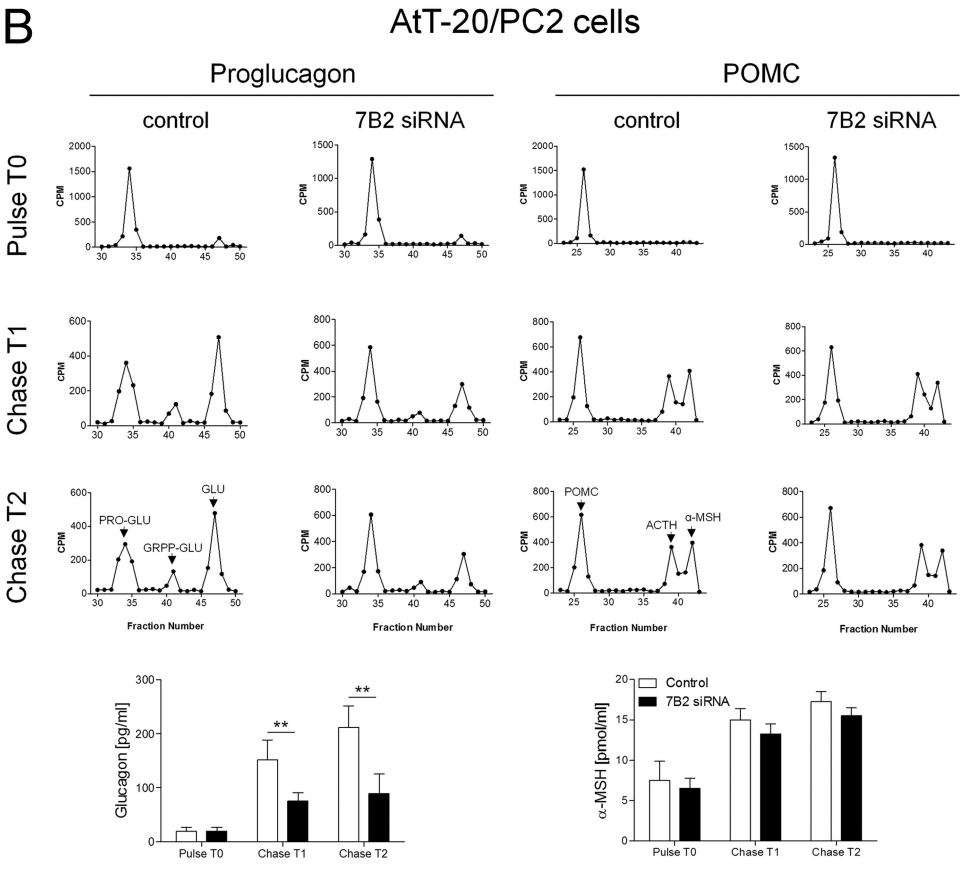
The relative abundance of endogenous 7B2 within the various cell lines used (prior to adenovirus- or RNAi-mediated manipulation of 7B2 levels) was comparable, as determined by radioimmunoassay, with levels ranging from 25 to 35 fmol/100 μl assayed sample (one-tenth of the total of a 6-well plate) (supplemental Fig. S1). This observation is in line with Northern blot analysis in a previous study that detected comparable 7B2 levels in these same cell lines (32).

In AtT-20/PC2 Cells and RinPE Cells, 7B2 Adenoviral Infection Increases Secretion of Active PC2 but Does Not Enhance Precursor Processing—Despite this increase in secreted PC2 activity, no significant increase in either cellular, basal, or stimulation-dependent secreted α-MSH was detected (Fig. 4*A*, *top*); nor was there a change in cellular or secreted ACTH in AtT-20/PC2 cells (Fig. 4*A*, *bottom*). Application of 7B2 siRNA to AtT-20/PC2 cultures also failed to reduce cellular α-MSH levels (Fig. 4*B*).

Adenoviral infection experiments were also performed in RinPE cells. Fig. 3*C* shows that adenoviral 7B2 overexpression also enhanced the release of PC2 activity in this cell line. In metabolic labeling studies, no alteration in the processing of proenkephalin to the heptapeptide Met-enkephalin-Arg-Phe, a PC2-mediated process (24), was evident following overexpression of 7B2 (supplemental Fig. S2) despite the presence of ample quantities of the PC2 substrate Peptide B. These results show that in AtT-20/PC2 and RinPE cells, 7B2 overexpression generates large increases in secreted PC2 activity, but this influences neither cellular precursor processing nor the releasability of stored peptides.

In α-TC6 Cells, Modulation of 7B2 Levels by Adenoviral Infection or by siRNA Transfection Alters the Production of Glucagon—A previous report using mouse strains expressing different 7B2 levels had suggested that glucagon levels were


A
α-TC6 cells
Proglucagon
POMC
control
7B2 siRNA
control
7B2 siRNA
Pulse T0
Chase T1
Chase T2
CPM
PRO-GLU
GLU
GRPP-GLU
POMC
ACTH
α-MSH
Fraction Number
Glucagon [pg/ml]
α-MSH [fmol/ml]
Control
7B2 siRNA
Pulse T0
Chase T1
Chase T2


B
AtT-20/PC2 cells
Proglucagon
POMC
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7B2 siRNA
control
7B2 siRNA
Pulse T0
Chase T1
Chase T2
CPM
PRO-GLU
GLU
GRPP-GLU
POMC
ACTH
α-MSH
Fraction Number
Glucagon [pg/ml]
α-MSH [pmol/ml]
Control
7B2 siRNA
Pulse T0
Chase T1
Chase T2

directly correlated with 7B2 expression levels (22); we therefore decided to investigate this precursor system. Adenoviral infection of α-TC6 cells with 7B2-encoding virus increased 7B2 levels 2.5-fold (Fig. 5*A*). Immunoreactive glucagon also increased by 50% (Fig. 5*B*), confirming that in α-TC6 cells, increased 7B2 expression correlates directly with increased bioactive peptide production. When 7B2 siRNA was added to α-TC6 cells, 7B2 levels dropped by 62% (Fig. 5*C*); immunoreactive glucagon levels also decreased by 50% (Fig. 5*D*). These results indicate that in α-TC6 cells, 7B2 levels are rate-limiting for glucagon production.

In POMC-expressing α-TC6 and Glucagon-expressing AtT-20/PC2 Cells, Knockdown of 7B2 by siRNA Transfection Lowers the Production of Glucagon but Does Not Affect POMC Processing—The above results show that although POMC processing is not altered by modulation of 7B2 expression in AtT-20/PC2 cells, proglucagon processing is affected in α-TC6 cells. To determine whether the 7B2-mediated effect on proglucagon processing is cell type- or precursor-specific, we transfected α-TC6 cells (endogenously expressing proglucagon) with plasmid DNA encoding POMC. We also transfected AtT-20/PC2 cells (endogenously expressing POMC) with plasmid DNA encoding proglucagon. This was followed by RNAi-mediated knockdown of 7B2 in both cell lines (Fig. 6).

7B2-dependent changes in the generation of proglucagon- and POMC-derived peptides in α-TC6 (Fig. 6*A*) and AtT-20/PC2 (Fig. 6*B*) cells were analyzed by pulse-chase experiments using [^{35}S]methionine/cysteine followed by immunoprecipitation. Fig. 6 (*top panels*) shows that labeled proglucagon (*left*) and POMC (*right*) species in the two cell lines are present both as the intact precursor and as smaller cleaved forms. Comparison of the peak intensities of the various labeled glucagon forms indicates that the ratio of cellular proglucagon to cleaved products increased when 7B2 was knocked down, whereas at the same time, levels of smaller proglucagon-derived peptides decreased. A decrease in proglucagon-derived products (and an increase in proglucagon) were also observed when pulse-chase samples were analyzed using SDS-PAGE and phosphorimaging following 7B2 knockdown (supplemental Fig. S3). In addition, quantification of glucagon concentrations within the pulse-chase samples by RIA showed that glucagon levels decreased by ~50% *versus* controls when 7B2 was knocked down, in both α-TC6 and AtT-20/PC2 cells (Fig. 6, *A* and *B*, *bottom panels*).

By comparison, cleavage patterns of POMC were only slightly altered in response to the RNAi-mediated decrease of 7B2 (Fig. 6, *A* and *B*, *top panels*). There was no significant change in cellular levels of POMC and no discernable effect of lowered 7B2 levels on the production of POMC-derived peptides. This observation was confirmed by measuring cellular α-MSH concentrations using an α-MSH-specific RIA in 7B2-RNAi and control chase samples (Fig. 6, *A* and *B*, *bottom panels*).

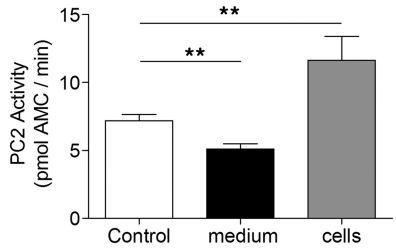

FIGURE 7. **Decreased PC2 activity is secreted from α-TC6 cells following 7B2 overexpression by adenoviral transduction, whereas cellular PC2 activity is increased.** α-TC6 Cells were infected for 24 h with control adenovirus or adenovirus encoding 7B2 for 24 h followed by incubation in Opti-MEM for 18 h; conditioned medium and cell extracts were collected. Activity of PC2 in the medium and in cell extracts was measured in the presence of protease inhibitors and with and without the 7B2 C-terminal peptide to assess specificity. Overexpression of 7B2 was accompanied by decreased PC2 activity in the medium, whereas 7B2 overexpression increased the amount of active PC2 within cells. Data represent $n = 3$ wells/group, mean ± S.D.; one-way ANOVA; **, $p > 0.01$ (*error bars*).

In summary, glucagon production was affected by 7B2 manipulation in both cell lines, whereas processing patterns of POMC were only slightly altered. Thus, these results indicate distinct precursor specificity for 7B2-mediated effects.

Furthermore, we tested whether the storage and secretion patterns of active PC2 in α-TC6 cells differ from those observed in AtT-20/PC2 cells. This was accomplished by measuring enzymatic activity of secreted and cellular PC2 following adenovirus-induced overexpression of 7B2. Fig. 7 shows that in α-TC6 cells, adenoviral overexpression of 7B2 decreased the release of active PC2, in direct contrast to results obtained in AtT-20/PC2 cells, where adenoviral infection increased the amount of active PC2 secreted (Fig. 3*B*). At the same time, however, intracellular PC2 activity was increased (Fig. 7), possibly accounting for the enhanced amounts of mature glucagon observed in Fig. 5*B*.

Up-regulation of 7B2 in Congenic B62D-3 F2 cast/cast Mice Is Accompanied by Increased Levels of Blood Glucagon and Glucose Levels—Congenic B62D-3 F2 mice heterogeneous for certain alleles (known as "*cast*" alleles) within a specific domain of chromosome 2 harboring the 7B2 locus have been demonstrated to differentially express 7B2. Mice homozygous for the *cast* alleles in this region express nearly 3 times more 7B2 mRNA and protein than mice with *b6* alleles in this region, whereas heterozygous mice are intermediate expressers (29).

FIGURE 6. **RNAi-mediated knockdown of 7B2 in POMC-expressing α-TC6 and proglucagon-expressing AtT-20/PC2 cells decreases the production of cellular glucagon, whereas levels of POMC and its products are unaltered.** α-TC6 cells were transfected with POMC, and AtT-20/PC2 cells were transfected with preproglucagon followed by knockdown of 7B2 with siRNA. Cells were pulse-labeled with [^{35}S]Met/Cys and chased for the indicated time periods. Immunoprecipitates of glucagon- and POMC-related products were size-separated by high performance gel permeation chromatography, and selected fractions were subjected to quantification by scintillation counting (*top panels*). Additionally, glucagon- or α-MSH-specific RIAs were utilized to quantify the levels of cleavage products in the pulse/chase samples (*bottom panels*). Metabolic labeling showed decreased conversion of proglucagon into smaller cleavage products when 7B2 was knocked down in α-TC6 (*A*, *top left*) and AtT-20/PC2 cells (*B*, *top left*). Quantification of cleaved glucagon levels in pulse-chase samples by RIA revealed decreased concentrations of mature glucagon after treatment with 7B2 siRNA (*bottom left panels*). Cleavage patterns of POMC in both cell lines were largely unaltered following 7B2 knockdown (*top right panels*); there was also no difference in the production of α-MSH within the samples as determined by RIA (*bottom right panels*). Data represent $n = 2$ wells/group, each measured in triplicate, mean ± S.D. (*error bars*); one-way ANOVA; **, $p > 0.01$; ***, $p > 0.001$. *T0*, time point zero; *T1*, 1-h incubation; *T2*, 2-h incubation. *PRO-GLU*, proglucagon, *GRPP-GLU*, glicetin-related pancreatic polypeptide-glucagon fragment; *GLU*, glucagon.

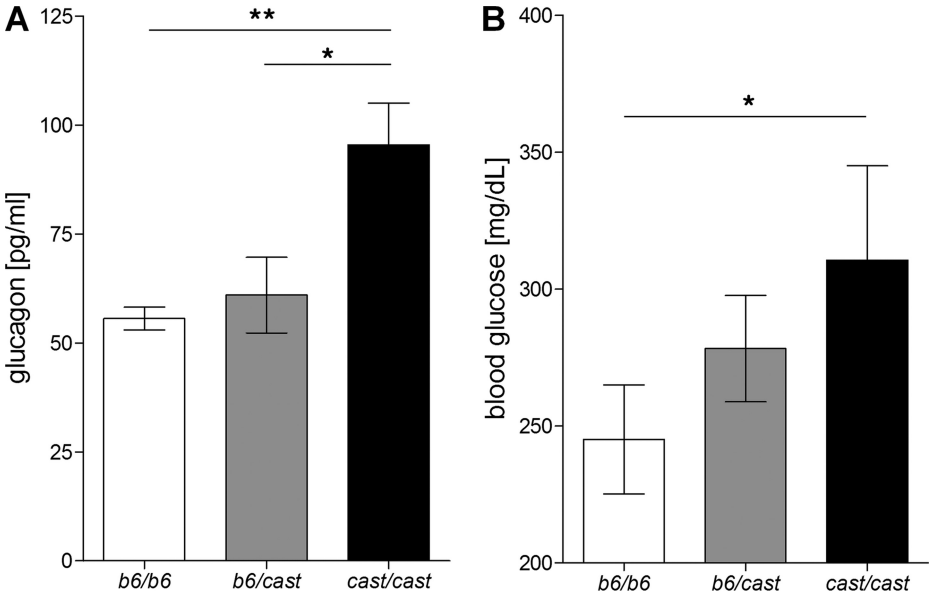

FIGURE 8. **Circulating glucagon and glucose levels are increased in 7B2-overexpressing B62D-3 F2 *cast/cast* mice.** Blood plasma glucagon and glucose levels of congenic B62D-3 F2 mice were measured by glucagon-specific RIA and with a handheld glucose meter in three genotypes with varying levels of 7B2 expression. With regard to levels of 7B2, *b6/b6* mice represent low 7B2 expressers ($n = 8$); *b6/cast* mice represent intermediate expressers ($n = 3$); and *cast/cast* mice represent high expressers ($n = 8$) (mean ± S.E. (*error bars*); one-way ANOVA; *, $p > 0.05$; **, $p > 0.01$).

Quantification of mature glucagon in blood plasma samples by RIA within these three genotypes revealed a significant 60% increase in the high 7B2-expressing *cast/cast* mice ($p = 0.008$) (Fig. 8*A*). Increased glucagon levels in *cast/cast* mice were accompanied by increased blood glucose levels in 7B2 overexpressing mice ($p = 0.034$) (Fig. 8*B*).

DISCUSSION

The small neuroendocrine protein 7B2, present in all neural and endocrine tissues, is now known to be required for the successful maturation of cellular proPC2 to an active form (reviewed in Refs. 13 and 14). More recent work has shown that 7B2 acts to block the aggregation of proPC2 into unactivatable forms (15). 7B2 may subserve additional functions, because it is present in cells lacking PC2 (32) and also circulates in blood (14, 21). In the intermediate lobe of the 7B2 null mouse pituitary, the lack of 7B2 results in the absence of PC2 activity. This eliminates an important PC2-mediated step (4) in the processing of proopiomelanocortin, the cleavage of intact adrenocorticotropic hormone 1–39 to adrenocorticotropic hormone 1–14, the precursor of α-MSH, and corticotropin-like intermediate lobe peptide (18). In 7B2 nulls, adenovirally expressed 7B2 decreases the intermediate lobe content of intact adrenocorticotropic hormone 1–39 and decreases adrenocorticotropic hormone hypersecretion (21), supporting the idea that 7B2 levels directly control adrenocorticotropic hormone processing in this tissue. However, stable overexpression of 7B2 in AtT-20/PC2 cells, a pituitary-derived cell line, did not result in increased adrenocorticotropic hormone processing to α-MSH, indicating that the correlation between 7B2 and adrenocorticotropic hormone processing does not hold in all cell systems. The goal of the present study was to elucidate the effects of 7B2 on precursor processing in several different neuroendocrine systems.

Similar to results found in intact null animals (18), our studies show that 7B2 adenoviral infection of primary pituitary cells obtained from 7B2 null mice results in elevated α-MSH levels. This indicates that 7B2 overexpression can also rescue proopiomelanocortin processing defects in primary tissue culture. However, the finding of enhanced precursor processing did not extend to the PC2-overexpressing cell line, AtT-20/PC2. The major effect we consistently observed as a consequence of infection of this cell line with 7B2-encoding adenovirus was greatly increased secretion of PC2 activity. Increased secretion of active PC2 caused by 7B2 introduction was also observed by Seidel *et al.* (11) in SK-N-MCIX cells, the only neuroendocrine cell line that does not endogenously express 7B2; peptide precursor processing was not examined in this study. However, in AtT-20/PC2 cells, increased release of active enzyme was not accompanied by increased precursor processing. Similar results were observed in RinPE cells, in which 7B2 also did not appear to represent a limiting factor for Peptide B conversion to Met-enkephalin-Arg-Phe, a known PC2-mediated event (24). We speculate that in AtT-20 and Rin cells, the additional active PC2 generated by 7B2 overexpression may not reach the secretory granule compartment (where its adrenocorticotropic hormone substrate is concentrated) but is instead secreted constitutively.

We also considered the possibility that the time required for complete processing of a given PC2 substrate is reached more quickly in some cell types (such as α-TC6) and for some substrates (*e.g.* proglucagon) than in other cells and for other substrates. The fact that adenoviral overexpression of 7B2 in α-TC6 cells resulted in increased processing of proglucagon accompanied by decreased secretion of active enzyme (rather than the increased secretion observed in AtT-20 cells) strongly implies that 7B2-mediated targeting of PC2 differs between various cell lines. This suggests that 7B2 overexpression can only result in increased precursor cleavage when the increased

active PC2 is actually targeted to secretory granules. However, we also found that various prohormone substrates can be differentially affected by 7B2 manipulation. Our proglucagon and proopiomelanocortin processing experiments in AtT-20 cells show that reduction of 7B2 levels by antisense lowered PC2-mediated proglucagon processing while leaving proopiomelanocortin processing entirely intact. These results agree with previous proopiomelanocortin processing results in 7B2-overexpressing AtT-20 cells (31) and results obtained *in vivo*, where the increased 7B2 level present in *cast/cast* mice has no effect on circulating or pituitary α-MSH (29). Our present observation of increased glucagon in *cast/cast* mice also supports the finding by Schmidt *et al.* (22) that circulating glucagon levels correlate positively with differing pancreatic 7B2 expression in various mouse strains. Thus, manipulation of 7B2 levels appears to more profoundly affect processing of proglucagon than other precursors. One possible explanation for this effect is a relatively enhanced susceptibility of the proglucagon precursor and/or its glucagon-containing intermediates to PC2-mediated cleavage. Indeed, our pulse-chase results show that not only glucagon but also proglucagon is affected by 7B2 antisense introduction. Preliminary experiments using purified peptides also show that the glucagon precursor oxyntomodulin is more easily cleaved by PC2 than is the α-MSH precursor adrenocorticotropic hormone. It will be interesting in future work to compare the relative susceptibility of other pancreatic PC2-cleaved precursors, such as islet amyloid polypeptide (33) and proinsulin, to manipulation of 7B2 levels to determine whether the profound effect of 7B2 on proglucagon processing is unique. If so, this might bode well for the development of therapeutic PC2 inhibitors targeted toward the reduction of pancreatic and circulating glucagon in diabetes.

In conclusion, our data indicate that 7B2 expression represents an important factor in the control of peptide processing. These results are of interest in light of the fact that differences in the 7B2 promoter have been shown to affect its expression both in mice (22, 29) and in humans (34). Genetic polymorphisms in the 7B2 promoter have been associated with glucose intolerance (35), supporting the physiological relevance of alterations in 7B2 expression. Because large reservoirs of proPC2 are stored in the endoplasmic reticulum of many PC2-expressing cells (36, 37), dynamic regulation of 7B2 levels could potentially result in a relatively rapid and selective increase in cellular production of bioactive peptide.

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