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Sorting of growth hormone-erythropoietin fusion proteins in rat salivary glands

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Abstract

Neuroendocrine and exocrine cells secrete proteins in either a constitutive manner or via the regulated secretory pathway (RSP), but the specific sorting mechanisms involved are not fully understood. After gene transfer to rat salivary glands, the transgenic model proteins human growth hormone (hGH) and erythropoietin (hEpo) are secreted primarily into saliva (RSP; exocrine) and serum (constitutive; endocrine), respectively. We hypothesized that fusion of hGH at either the C-terminus or the N-terminus of hEpo would re-direct hEpo from the bloodstream into saliva. We constructed and expressed two fusion proteins, hEpo-hGH and hGH-hEpo, using serotype 5-adenoviral vectors, and delivered them to rat submandibular glands in vivo via retroductal cannulation. Both the hEpo-hGH and hGH-hEpo fusion proteins, but not hEpo alone, were secreted primarily into saliva ($p<0.0001$ and $P=0.0083$, respectively). These in vivo studies demonstrate for the first time that hGH, in an N- as well as C-terminal position, influences the secretion of a constitutive pathway protein.

INTRODUCTION

Sorting mechanisms of secretory proteins are not fully understood. The prevailing paradigm describes two general pathways for sorting of secretory proteins in polarized cells[1,2]. In the first, proteins are secreted as fast as they are synthesized, in a constitutive, non-directional manner and independent from changes in second messenger levels. In the second pathway, the regulated secretory pathway (RSP), proteins destined for secretion are sorted and stored in high concentrations in secretory granules where they await an external secretory stimulus[3–5]. RSP proteins require an amino acid-based sorting signal and evidence supports both the “sorting-for-entry” and “sorting-by-retention” hypotheses for these proteins[6–10]. At present no universal sorting signals for secretory proteins have been identified in any cell type. Additionally, most studies of secretory protein sorting have been performed in transformed cell lines in vitro, with few studies performed in vivo or with acutely prepared primary cells [8,11–14]. However, it is clear that specific proteins can be sorted differently in various cell types, and transformed cell lines may exhibit dramatically different secretory protein sorting behaviors than primary cells or the native cells in situ[8,13].

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Several distinct protein secretion pathways have been identified in salivary gland cells, within the general constitutive and RSP categories, including both major and minor regulated pathways, apical and basolateral constitutive pathways, and a constitutive-like pathway[9, 15]. These lead to specific sorting routes for transgenic secretory proteins in an endocrine and exocrine manner [16]. For example, human growth hormone (hGH), which in the anterior pituitary gland is secreted into the bloodstream via the RSP, when expressed from an adenoviral vector delivered to rodent and miniature pig salivary glands, follows the tissue specific RSP, i.e., the exocrine route into saliva[17–19]. Conversely, transgenic human erythropoietin (hEpo) is secreted from murine salivary glands almost entirely via an endocrine route into the bloodstream [20–22]. Ideally, control over protein entry into either of these general pathways could provide a means for selectively directing transgene products to a desired site, i.e., either into the bloodstream or into saliva, as appropriate for clinical applications.

To help understand signals involved in RSP sorting in vivo, we tested two hEpo fusion proteins in rat submandibular glands after gene transfer. In addition to testing a hEpo-hGH construct previously examined in mice [23], we also constructed a new fusion protein with hEpo at the C-terminus of hGH (hGH-hEpo). We hypothesized that both constructs could re-direct immunoreactive hEpo into the exocrine pathway, i.e., to saliva.

MATERIALS AND METHODS

Plasmids

Coding sequences of hEpo and hGH were fused with PCR using splicing by overlap extension (Advantage HF PCR kit from BD biosciences Clontech)[24]. Oligonucleotides spanning the desired fusion site and encoding *Eco*RI and *Bam*HI were designed (for the hEpo-hGH construct, hEpo, F1 5′-CGGAATTCCGATGGGGGTGCACGAATG-3′and R1 5′-GGGAGCCTGCAGCCATTCTGTCCCCTGTCCTGCA-3′;hGH, F2 5′-ATGGCTGCAGGCTCCC-3′and R2 5′-CGCGGATCCGCGCTAGAAGCCACAGCTGCC-3′; for the hGH-hEpo construct, hGH, F1 5′-CGGAATTCCGATGGCTGCAGGCTCCC-3′and R1 5′-ATTCGTGCACCCCCATGAAGCCACAGCTGCCC-3′ ;hEpo, F2 5′-ATGGGGGTGCACGAAT-3′and R2 5′-CGCGGATCCGCGTCATCTGTCCCCTGTCCTG-3′). These primers were then used to produce two initial PCR products for each construct. The products were next hybridized with their respective flanking primers (F1 and R2) to produce the full-length chimeric cDNA of hEpo-hGH (hEpo fused to the 5′ end of hGH) and hGH-hEpo (hGH fused to the 5′ end of hEpo). PCR products were directionally cloned into the expression vector pAC-CMV-pLpA and the resultant plasmids, pAC-CMV-hEpo-hGH and pAC-CMV-hGH-hEpo were sequenced and confirmed to contain the correct coding sequences.

Recombinant adenoviral vector

First-generation, E1^{-}, recombinant adenoviruses (serotype 5) encoding either hEpo (AdCMVhEpo), hEpo-hGH (AdCMVhEpo-hGH), hGH-hEpo (AdCMVhGH-hEpo), or hGH (AdCMVhGH) were constructed as previously reported[25] by contransfection of the shuttle plasmid pAC-CMV-pLpA with the corresponding transgenes, together with the adenoviral plasmid pJM17 into 293 human embryonic kidney (HEK) cells. The vectors were amplified in HEK 293 cells and purified by two rounds of CsCl gradient centrifugation as described [26]. Purified vectors were dialyzed against 4 L of dialysis buffer containing 10% glycerol, 0.1 *M* Tris (pH 7.4), 5 m*M* $MgCl_2$, for 4 h at 4°C and stored in aliquots at −80°C for later use. Vectors were titered by quantitative PCR (ABI Prism 7700, Applied Biosystems, Foster City, CA) with primers from the E2 region of adenovirus, E2q1 (5′-

GCAGAACCACCAGCACAGTGT-3′) and E2q2 (5′-TCCACGCATTTCCTTCTAAGCTA-3′).

Cell transduction and Western blot analysis

Correct expression of the hEpo-hGH and hGH-hEpo fusion proteins was evaluated in HEK 293 cells. Cells were grown in IMEM (improved minimal essential medium, Eagle's) supplemented with 10% bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin (all from Biosource, Camarillo, CA) at 37°C in a humidified 5% CO_2 atmosphere incubator. Cells were transduced with 100 particles/cell of AdCMVhEpo, AdCMVhEpo-hGH or AdCMVhGH-hEpo, in 6-well plates. 48 h after transduction the conditioned medium (without serum) was collected, centrifuged at 3000 rpm for 3 min and used in ELISAs for hEpo and hGH (see below). The cells were then rinsed with PBS, harvested, lysed with M-PER protein extraction reagent (Pierce), centrifuged at 1000 g for 10 min to remove debris and the soluble extract was used for Western blot analysis [23].

Vector delivery to rat salivary glands

Animal experiments were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee and the National Institute of Health Biosafety Committee. Male Wistar rats (~250–350 grams) were anesthetized with a mixture of 60 mg/ml ketamine (Phoenix Scientific, St. Joseph, MO) and 8 mg/ml xylazine (Phoenix Scientific) given intramuscularly (1 μl/g of body weight) followed by cannulation of submandibular gland ducts with modified polyethylene tubing (Intramedic PE-10, BD Diagnostic Systems, Sparks, MD). Atropine (i.m., 0.5 mg/kg body weight; Sigma, St. Louis, MO) was administered to decrease salivary flow and after 10 min, 10^{10} viral particles/gland (200 μl volume) of either AdCMVhEpo, AdCMVhEpo-hGH, AdCMVhGH-hEpo or AdCMVhGH were administered by retrograde ductal delivery into the submandibular glands. After 72 h, rats were anesthetized and given a subcutaneous injection of pilocarpine to induce saliva secretion (0.5 mg/ml, 1 μl/g body weight; Sigma, St. Louis, MO). Whole saliva and blood (retro-orbital vein) were collected and following separation of serum were stored at −80°C until assayed for hEpo using a specific ELISA (see below).

In vivo distribution of secretory proteins

The concentration of each secretory protein was determined in saliva and serum samples using hEpo (Stemcell technologies, Vancouver, Canada) or hGH (Anogen, Mississauga, Ontario Canada) ELISAs. The distribution ratio (saliva to saliva plus serum) was calculated on the basis of the total amount of transgene product found in each fluid. For salivary calculations, we utilized an average stimulated salivary fluid secretion volume of 150 ul [27]. Thus, each measured salivary transgene protein concentration, in mU per milliliter for hEpo ELISAs (or nanogram per milliliter for hGH ELISAs), was multiplied by 0.15 to yield the total amount of the protein found in saliva. For calculating total serum levels, we multiplied the determined concentration by 4 (the approximate volume of serum in mL) [17].

Data from in vivo experiments were analyzed with SigmaStat software (version 2.03; Systat Systems, Point Richmond, CA), using the Mann-Whitney test, and are presented, unless otherwise stated, as means ± SEM. Differences with $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Media from HEK 293 cells transduced with AdCMVhEpo-hGH and AdCMVhGH-hEpo reacted positively with both the hEpo and hGH ELISAs (Fig. 1). This validated the transduction ability and function of the experimental vectors we constructed, since both ELISAs are specific

for their antigens. Furthermore, proteins from the cell lysates analyzed by Western blot, immunoreacted with anti-hEpo antibody and showed the expected size of ~56 kDa for both hEpo-hGH and hGH-hEpo fusion proteins (Fig. 2). The apparent mass of hEpo and hGH are ~34 and ~22 kDa (not shown), respectively. Additionally, an immunoreactive band (~110 kDa) was also occasionally detected. The 110kDa band likely represents the dimeric form of the fusion protein. The lack of this dimeric form in the hGH-hEpo samples would suggest that the C-terminal of hGH might be involved in this process. Indeed Glu (174) in the C-terminal α-helix has been identified as an important residue for hGH dimerization [28]. In additional experiments these products were shown to be glycosylated as they migrated at a lower molecular mass when treated with 2,N-glycosidase to remove N-linked oligosaccharides (data not shown).

We next delivered both experimental vectors to rat submandibular glands *in vivo*. 72 h following vector administration serum and saliva were collected and assayed for hEpo levels. The ratio of the total amount of immunoreactive hEpo in saliva to that found in saliva plus serum are presented for different constructs in Figure 3. Higher ratios indicate secretion of the construct preferentially into saliva, i.e., via an exocrine route. While the ratios of animals administered AdCMVhEpo had a mean value of 0.396 (median 0.343), those of AdCMVhEpo-hGH and AdCMVhGH-hEpo had mean values of 0.886 and 0.808, respectively (median 1 and 1). The differences in these values between the control vector (AdCMVhEpo) and the experimental groups were highly statistically significant ($p<0.0001$ and $p=0.0083$). The median values of the various hEpo constructs secreted into saliva and serum, as well as those for hGH, are presented in Table 1. Thus, while animals administered AdCMVhEpo secreted immunoreactive hEpo predominantly into serum as expected, animals that received either AdCMVhEpo-hGH or AdCMVhGH-hEpo secreted the immunoreactive transgene products (hEpo-hGH or hGH-hEpo) almost exclusively into saliva (Fig. 3).

Although no universal regulated pathway-sorting motifs or signals have been identified [7,8], studies of prohormone/proneuropeptide trafficking have provided evidence that sorting of these molecules to the RSP involves a receptor-mediated mechanism[29,30]. For example, in endocrine cells, sorting motifs of pro-opiomelanocortin (POMC), brain derived neurotrophic factor and proinsulin were shown to interact with membrane carboxypeptidase E, which acts as a sorting or retention receptor to target these prohormones to the RSP[31]. hGH contains a highly conserved C terminus domain that is similar to the RSP sorting domain of POMC. Indeed, it was previously shown in vitro that fusion at the C-terminus of hGH to a model constitutive pathway viral protein (a truncated vesicular stomatitis virus G sequence), redirected the viral protein into the regulated pathway in the AtT20 model endocrine cell line [32]. Our earlier studies have suggested that the C-terminus of hGH at least in part can influence hGH sorting and secretion from rat submandibular glands[33]. However, as shown herein, fusion of hEpo to hGH, at either the N- or C-termini, results in a significant re-direction of immunoreactive hEpo from serum into saliva (Fig. 3), i.e., both hEpo-hGH and hGH-hEpo primarily enter saliva unlike the native hEpo. Given that hGH in either configuration studied by us exerts a strong influence on hEpo secretion, it seems reasonable to suggest that additional domains within hGH, including the N-terminus, may influence hGH sorting.

In the present study performed in rats we observed a significant difference in the secretion of hEpo-hGH compared to that we previously reported in mice [23], i.e., the redirection of hEpo-hGH into saliva was dramatically more efficient in rats. This is surprising given the morphological and functional similarities of the submandibular glands in these two species [34], but the results herein, obtained with large numbers of animals, indicate differences in the intracellular sorting machinery of these two rodent models. Our findings, thus have given rise to a model system in which components of the sorting process may be identified by comparison of the differences in mice and rats.

In conclusion, the present study in male rats shows that a normally constitutively secreted protein, hEpo, can be re-sorted from an endocrine into an exocrine secretory pathway of salivary gland cells in vivo, and this appears influenced by hGH in an N- as well as C-terminal position. Future studies to elucidate the specific sorting motifs and intracellular molecules involved should facilitate the use of salivary glands as a gene transfer target site for clinical applications.

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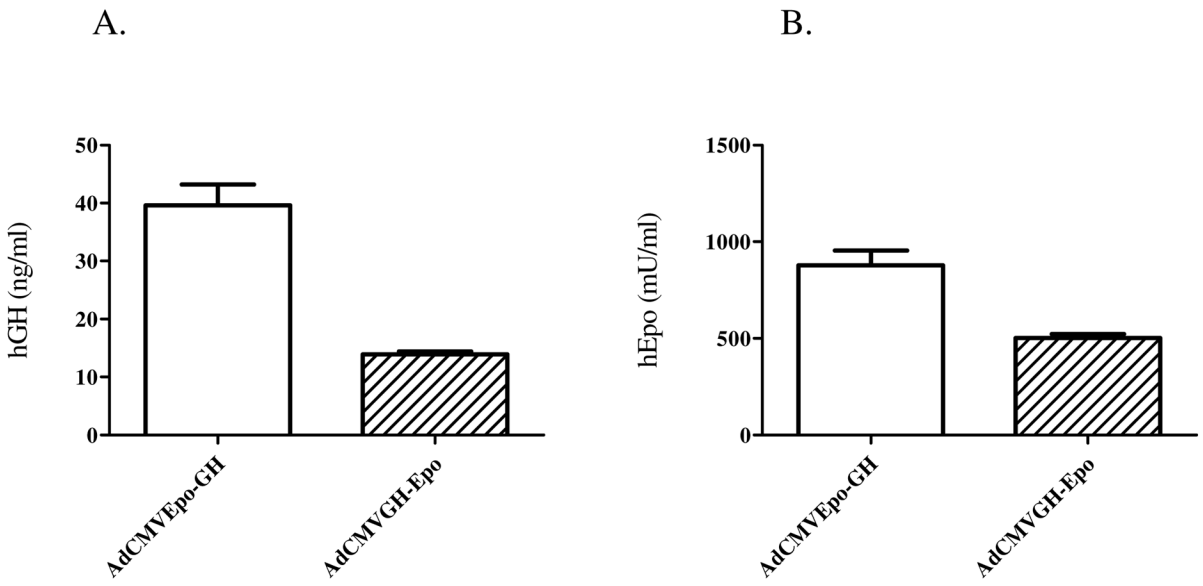

Fig. 1. Immunoreactive hGH and hEpo detected in media of transduced HEK 293 cells

Forty-eight hours following transduction with either AdCMVhEpo-hGH or AdCMVhGH-hEpo, media were collected and assayed with hGH (A) or hEpo (B) ELISAs. Results demonstrate positive immunoreactivity of hGH-hEpo and hEpo-hGH with both hEpo and hGH ELISAs. Data are presented as means ± SEM (n=3).

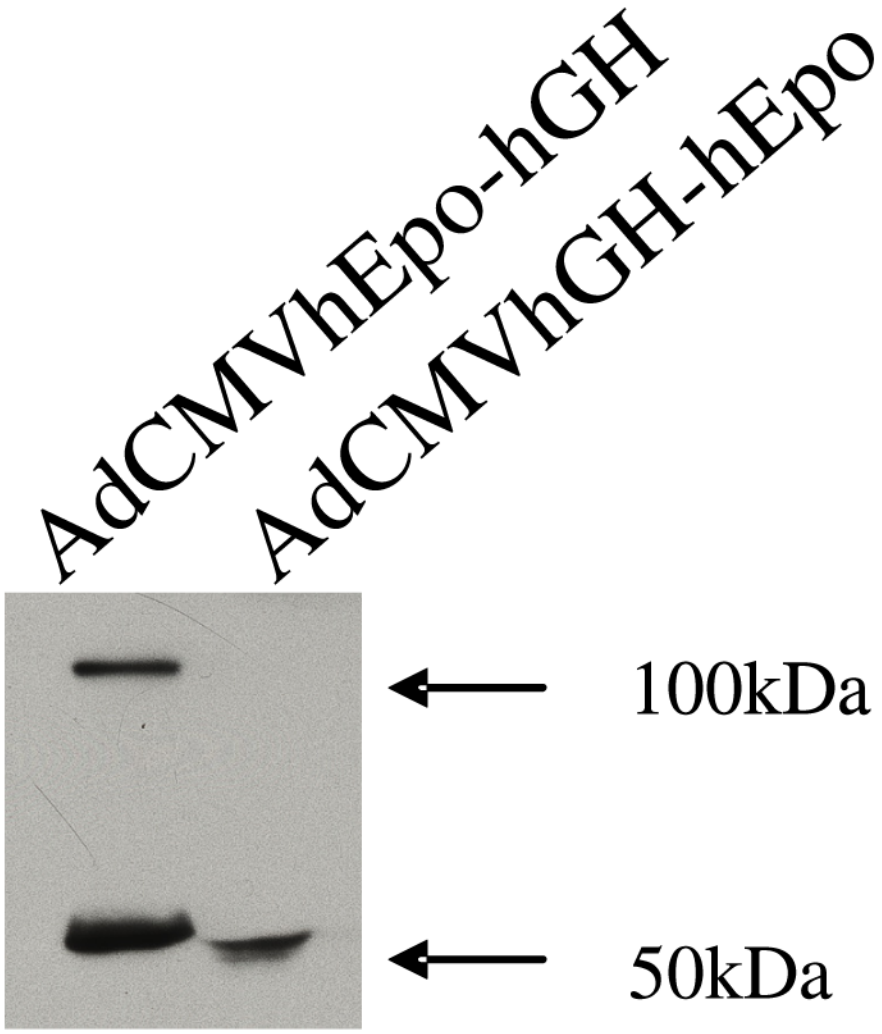

Fig. 2. Western blots from lysates of transduced cells

Lysates of cells transduced with AdCMVhEpo-hGH or AdCMVhGH-hEpo, at 100 particles/cell, were electrophoresed and reacted with anti-hEpo antibody. The predicted size of the chimeric hEpo-hGH and hGH-hEpo fusion proteins is ~56 kDa. The arrows to the right indicate the molecular masses of simultaneously electrophoresed protein standards. See Materials and Methods, and Results, for additional details.

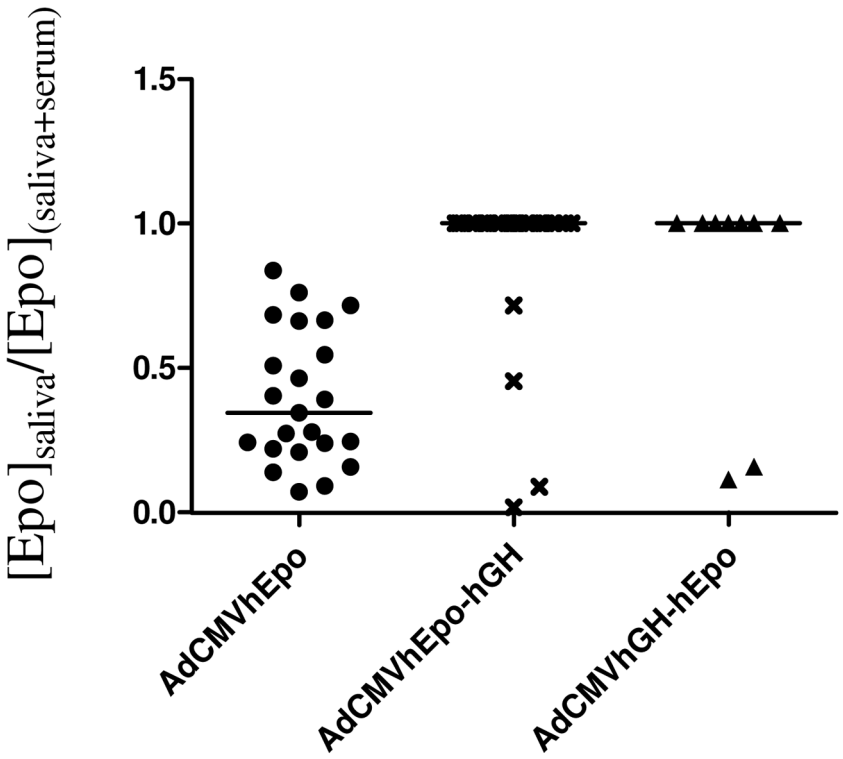

Fig. 3. Ratios of immunoreactive hEpo products in saliva to that in saliva + serum

Rats were administered vector to their submandibular glands. 3 days later serum and saliva were collected and hEpo immunreactive products measured as described in Materials and Methods. Animals transduced with AdCMVhEpo-hGH, or AdCMVhGH-hEpo had statistically higher saliva/saliva plus serum ratios of immunoreactive hEpo products when compared to those found in animals transduced with AdCMVhEpo ($p<0.0001$ and $p=0.0083$, respectively), i.e. relatively higher levels of immunoreactive hEpo were being secreted into saliva. For these analyses, the number of rats tested was for hEpo (n=23), for hEpo-hGH (n=24), and for hGH-hEpo (n=9). The horizontal bar represents the median value.

Table 1

Immunoreactive transgenic proteins secreted into rat saliva and serum 10^{10} viral particles were delivered to submandibular glands

Three days later, saliva and blood were collected for measurements. Total immunoreactive hEpo (in mU) or hGH (in ng) was calculated in 4 mL of serum and 0.15 mL of saliva, and median values are shown. The number (n) of animals tested with each construct is given in parentheses

	Saliva	Serum
hEpo (n=33)	50.61	132.4
hEpo-hGH (n=34)	19.99	0
hGH-hEpo (n=9)	6.55	0
hGH (n=5)	10.09	0