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***In vivo* secretion of the mouse immunoglobulin G Fc fragment from rat submandibular glands**

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Abstract

Background—Salivary glands have been proposed as target organs for gene therapy. They secrete endogenous, as well as transgenic proteins, in a polarized manner. Transgene-encoded regulated pathway proteins primarily follow the regulated pathway in rat salivary glands and are secreted into saliva in an exocrine manner. Conversely, constitutive pathway proteins generally are secreted more basolaterally and thus follow the endocrine route. In the present study, we studied *in vivo* the sorting of the mouse immunoglobulin G2b Fc fragment, which is physiologically secreted via the constitutive pathway.

Methods—Adenoviral vectors encoding the Fc fragment and human growth hormone were delivered into rat and mouse submandibular glands *in vivo* to compare their serum-to-saliva distribution. We also compared the intracellular localization of the Fc fragment and growth hormone by confocal microscopy.

Results—We found that the Fc fragment was secreted almost entirely into the bloodstream from rat and mouse submandibular glands via a constitutive or constitutive-like pathway. This sorting behaviour is clearly different from that of transgenic human growth hormone, which is secreted in a regulated pathway, both in neuroendocrine cells and as a transgenic protein from salivary gland cells. We also found that simultaneously expressed human growth hormone and the mouse Fc fragment do not appear to influence each other's sorting behaviour. The Fc fragment showed a primarily basal localization, whereas growth hormone showed an apical localization, in rat submandibular gland acinar cells.

Conclusions—The results obtained in the present study indicate that the mouse Fc fragment is a useful model protein for examining the basolateral versus apical secretory pathways employed by transgenic secretory proteins in salivary glands.

Keywords

basolateral secretion; constitutive pathway; gene therapy; salivary glands; sorting

Introduction

Salivary glands have been proposed as suitable target organs for gene therapy [1,2]. Salivary glands are easily accessible by cannulation, are not critical for life and can produce large amounts of proteins. Salivary gland epithelial cells are polarized, and secretion from these cells into the saliva can occur in an exocrine manner [1,3] or basolaterally into the bloodstream [1,

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4]. Exocrine secretion can be used to deliver therapeutic proteins to the upper gastrointestinal tract, whereas the endocrine route can be used for systemic protein delivery. The route of secretion depends on how the protein of interest is sorted within salivary gland cells.

Salivary acinar cells exhibit two regulated secretory pathways (RSPs) [5]. The major RSP involves the storage of select cargo proteins in large dense-core granules, which are discharged in response to high doses of β -adrenergic agonists, such as isoproterenol [6]. More than 80% of total protein secretion involves this pathway. In addition, a minor RSP also exists that originates in immature secretory granules [7]. In this pathway, smaller transport vesicles are secreted in response to muscarinic or weak β -adrenergic stimulation. Human growth hormone (hGH), which physiologically is secreted into the bloodstream from the anterior pituitary in a regulated manner, has been expressed in rat salivary glands [1], where it follows the exocrine RSP into the saliva. Hence, hGH can be used as a positive control for regulated secretion *in vivo* in salivary glands.

There are two different models to explain the selection of content proteins for storage in secretory granules [8]. According to the sorting-for-entry hypothesis, there are sorting signals on regulated proteins that are recognized by sorting receptors. To enter the forming secretory granule, a protein either has to be bound to a sorting receptor or it has to bind to other proteins that are already bound to the receptor. All other proteins are excluded from secretory granules. This sorting process takes place in the trans-Golgi network (TGN). The sorting-by-retention model, on the other hand, assumes that secreted proteins, unless directly or indirectly associated with the membrane, can freely enter the forming secretory granules, regardless of whether or not they are stored [8]. RSP proteins are retained and stored, whereas inefficiently stored proteins are then progressively removed from the maturing granules. According to this model, the immature secretory granule serves as an important post-TGN sorting station.

Salivary gland acinar cells also exhibit secretory pathways that do not depend on extracellular stimulation [5]. The constitutive pathway originates in the TGN and transports nongranule proteins [6]. The constitutive-like pathway originates in maturing secretory granules, does not require stimulation, and carries proteins that are poorly retained in large secretory granules during maturation [5,6].

Constitutively secreted proteins can be sorted apically or basolaterally [5]. Human erythropoietin (hEPO) is a constitutively secreted protein, physiologically produced in the kidney, which has been expressed in the salivary glands of mice [9–12] and rats [12–14]. Importantly, there is a difference in the sorting of transgenic hEPO when expressed in rat and mouse submandibular glands [12]. When considering the total amount of transgenic protein produced, hEPO is secreted into serum at high levels in both species. However, whereas very little transgenic hEPO is secreted into mouse saliva, in rat saliva, the concentration of hEPO is higher than it is in serum. Accordingly, the serum-to-saliva ratio of total secreted hEPO is 180 : 1 in mice and 11.5 : 1 in rats [12]. The reason for this species specific difference in hEPO sorting is unclear, but it indicates that *in vivo* sorting of the same protein may be different between species.

The sorting of immunoglobulin fragments has been studied *in vitro* [15,16]. In AtT20 cells, the Fc fragment enters secretory granules (i.e. the RSP) and its secretion can be stimulated. However, it is gradually removed from maturing secretory granules [15]. Thus, the Fc fragment behaved as a paradigm constitutive-like secreted protein in those *in vitro* experiments. However, the sorting of the transgenic Fc fragment has not been studied *in vivo* in a cell type containing a RSP. Therefore, the present study aimed to investigate: (i) the distribution of the Fc fragment in serum and in saliva after *in vivo* gene transfer to salivary glands; (ii) whether the serum-to-saliva distribution was affected by stimulation of salivary secretion; and (iii)

whether the secretion pattern of the Fc fragment was altered by co-expression of transgenic hGH.

Materials and methods

Animals

Male BALB/c mice and Wistar rats were obtained from Harlan-Sprague-Dawley (Walkersville, MD, USA) at 6–8 weeks of age. Animals were housed for at least 1 week before the experiments, with free access to food and water. Animal procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research and by the Biosafety Committee of the National Institutes of Health.

Vectors

A plasmid containing a prerenin signal peptide sequence followed by the Fc fragment of mouse immunoglobulin (Ig)G2b [15] was kindly provided by Drs Anna and David Castle (University of Virginia, Charlottesville, VA, USA) with the permission of Dr Tim Reudelhuber (Clinical Research Institute of Montreal, Canada). An E1 deficient, first generation, recombinant serotype 5 adenovirus encoding the secreted mouse Fc fragment was created essentially as described previously [17]. Briefly, the prerenin signal peptide-Fc insert was re-cloned into the shuttle plasmid pACCMV-pLpA containing a cytomegalovirus (CMV) promoter and a Simian virus 40 polyadenylation signal. The resulting pACCMV-preFc was verified to contain the insert and then it was cotransfected with the serotype 5 adenoviral helper plasmid pJM17 to Microbix 293 cells (Microbix Biosystems Inc., Toronto, Canada) to yield AdFc, which was then amplified in 293 cells and purified by two rounds of CsCl gradient centrifugation as described previously [18]. The AdhGH recombinant adenovirus serotype 5 vector, which encodes human growth hormone, was described previously [17].

Physical virus particle (vp) titers were determined by the real-time quantitative polymerase chain reaction (PCR). SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used in an ABI Prism 7700 Sequence Detector, with the conditions: 95 °C for 2 min, 95 °C for 8 min, 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The primers used amplified part of the CMV promoter: 5'-CAT CTA CGT ATT AGT CAT CGC TAT TAC-3' and 5'-TGG AAA TCC CCG TGA GTC A-3'. All samples and standards were assayed in triplicate.

In vitro transductions and immunoblotting

Microbix 293 cells were plated in modified Eagle's medium with Earle's salts (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/ml of penicillin G, 100 µg/ml of streptomycin and 2 mM glutamine (all from Invitrogen). Upon reaching confluence, cells were transduced overnight in serum-free medium with AdFc at a multiplicity of infection of ten viral particles per cell. The medium was then harvested and aliquots were stored at –80 °C until further processing. To detect the expression of the Fc fragment from media, aliquots of conditioned serum-free media were electrophoresed on NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen). Membranes were then immunoblotted with ECL Mouse IgG secondary antibodies (GE Healthcare, Piscataway, NJ, USA).

In vivo experiments

Animals were anesthetized with an intramuscular injection of a mixture of ketamine (60 mg/kg; Phoenix Scientific, St Joseph, MO, USA) and xylazine (6 mg/kg; Phoenix Scientific). Animals were given 0.5 mg/kg atropine (Sigma-Aldrich, St Louis, MO, USA) intramuscularly

10 min before vector delivery to decrease salivary flow. Submandibular glands were cannulated using modified PE-10 polyethylene tubing (BD Diagnostic Systems, Sparks, MD, USA) as described previously [14]. 10^8 , 10^9 or 10^{10} vp/gland were administered by retrograde infusion in 50 μ l of saline for mice and in 200 μ l of virus dilution buffer (10 mM TrisHCl pH 7.4, 0.1 mM MgCl₂, 10% glycerol) for rats. Syringes were left in place for 10 min after delivery.

After 48 h, animals were re-anesthetized as described above, and then given 0.5 mg/kg of pilocarpine subcutaneously and, as indicated, 5 mg/kg of isoproterenol intraperitoneally. Note that mice secrete negligible levels of 'resting' saliva [i.e. in the absence of parasympathetic (pilocarpine) stimulation]. Saliva was collected as described previously [4] and frozen on dry ice. Blood was collected by retro-orbital bleeding or by cardiac puncture, and serum was separated by centrifugation and stored frozen until use. Animals were sacrificed in a CO₂ chamber and death was confirmed by cervical dislocation. Submandibular glands were collected and frozen on dry ice until soluble gland extracts were prepared. Glands were homogenized using an Ultra-Turrax T25 device (IKA Works, Wilmington, NC, USA) in M-PER Mammalian Protein Extraction Reagent (4 ml per gland; Pierce, Rockford, IL, USA). Homogenates were centrifuged at 16 000 g and the resulting supernatants retained.

The mouse Fc fragment concentrations in rat samples were measured using a Mouse IgG2b enzyme-linked immunosorbent assay (ELISA) Kit (Alpha Diagnostic International, San Antonio, TX, USA). hGH concentration was measured with a Human Growth Hormone ELISA Kit (Anogen, Mississauga, Canada). The Fc fragment and hGH serum-to-saliva distribution ratio was calculated as described previously [1,19]. The Fc fragment in mouse saliva and serum samples was detected using 0.2- μ l aliquots of sera and 2 μ l of saliva by immunoblotting, as described above for the conditioned 293 cell media.

Immunofluorescence microscopy

Forty-eight hours after infusion of 10^9 vp each of AdFc and AdhGH together, rat submandibular glands were removed, and snap frozen in liquid nitrogen. Sections (20 μ m) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. To localize hGH and mouse Fc, slides were blocked in a solution containing 10% goat serum, 0.4% saponin and 0.02% sodium azide in PBS for 30 min. Free aldehydes were blocked by incubation with 100 mM glycine in PBS for 20 min. Slides were then incubated with a goat polyclonal anti-hGH antibody (sc-10 365, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1 : 100 dilution (2 μ g/ml) and an Alexa Fluor 488 conjugated donkey anti-mouse IgG (Invitrogen) at a dilution of 1 : 50 (40 μ g/ml) in 10% bovine serum albumin in PBS for 16 h at 4 °C. The slides were washed with PBS containing 100 mM glycine, and incubated with the secondary antibody (Alexa Fluor 546 conjugated donkey anti-goat IgG; Invitrogen) at 10 μ g/ml. After washing three times with PBS containing 100 mM glycine, sections were mounted in ProLong Gold antifade-4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen). As an internal control, an irrelevant rabbit IgG was used as a primary antibody on slides obtained from control rat submandibular glands infused with saline. Images were collected using a Leica Confocal microscope (Leica Microsystems, Wetzlar, Germany) and MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Data were analysed by the Kruskal–Wallis test and groups were compared by Dunn's post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Construction of an adenovirus encoding the mouse IgG2b Fc fragment

We constructed a first-generation serotype 5 adenovirus vector encoding the mouse Fc fragment (AdFc). Human embryonic kidney 293 cells were transduced overnight with AdFc at 10 vp/cell, and the secreted Fc fragment was detected in the medium as a protein of approximately 30 kDa by immunoblotting with an anti-mouse IgG antibody (Figure 1).

What is the serum-to-saliva distribution of Fc fragment secreted from rat and mouse submandibular glands?

To study dose-dependent Fc fragment secretion *in vivo*, we administered AdFc into rat submandibular glands at three different doses (10^8 , 10^9 or 10^{10} vp/gland). Forty-eight hours after administration, 0.5 mg/kg body weight of pilocarpine was injected subcutaneously to induce salivation. The Fc fragment levels in saliva, blood and transduced gland extracts were determined. As shown in Figure 2, the Fc fragment levels increased in gland extracts and serum with increasing amounts of AdFc administered. The Fc fragment was detected in saliva only after administration of 10^{10} vp AdFc, and the levels were quite low. Thus, the Fc fragment is secreted predominantly into the bloodstream from rat submandibular glands. For subsequent experiments, we chose to use the 10^9 vp dose as the one that resulted in relatively specific, and consistently detectable, Fc fragment secretion into serum, but not into saliva.

As mentioned above, transgenic hEPO is sorted differently from rat and mouse salivary glands [12]. To study how the Fc fragment was sorted in mouse submandibular glands *in vivo*, male BALBc mice were transduced in one gland with 10^9 vp of AdFc. Forty-eight hours after administration, 0.5 mg/kg body weight of pilocarpine was injected subcutaneously to induce salivation. Because the presence of endogenous mouse immunoglobulin precluded the use of a mouse IgG ELISA, western blotting was used to compare the level of the mouse Fc fragment in murine serum and saliva. Serum (0.2 μ l) and saliva (2 μ l) from each mouse was immunoblotted with anti-mouse IgG secondary antibodies. Transgenic Fc fragment expression was detected, at the appropriate molecular mass, in three of the five serum samples (mice 2, 3 and 4) but in none of the saliva samples (Figure 3). Thus, it appears that the Fc fragment also is secreted fairly specifically into the bloodstream from mouse submandibular glands.

Is there a difference in the sorting of the Fc fragment from rat submandibular glands after β -adrenergic stimulation?

Next, we compared the secretion of the mouse Fc fragment from rat submandibular glands after stimulating salivary flow with pilocarpine $\pm$ isoproterenol. 10^9 vp of AdFc were administered into single submandibular glands of male Wistar rats. After 48 h, saliva secretion was stimulated with 0.5 mg/kg body weight of pilocarpine either alone or in combination with 5 mg/kg body weight of isoproterenol. Pilocarpine is a parasympathomimetic agent that leads to minimal discharge of secretory granules at the same time as predominantly stimulating fluid secretion from salivary glands [6]. Isoproterenol, on the other hand, is a β -adrenergic agonist that discharges secretory granules and, when administered alone, induces a thick, protein-rich secretion [6].

Saliva, blood and submandibular glands were collected and Fc was measured in each sample with an ELISA. We then calculated the distribution of the Fc fragment detected (Figure 4A). In rats transduced with AdFc, the Fc fragment was secreted predominantly into the bloodstream. With pilocarpine stimulation, the median value of the total amount of the Fc fragment detected in serum was approximately 14.5%, with approximately 0.01% found in saliva and the remainder (approximately 85.5%) within the glands. In rats treated with pilocarpine plus isoproterenol, the median value of the total amount of the Fc fragment detected

in serum was approximately 20.5%, with approximately 0.4% found in saliva and the remainder (approximately 79%) within the glands. Thus, compared to pilocarpine alone, treatment of rats with pilocarpine plus isoproterenol did not significantly change the proportion of the Fc fragment found in sera, as well as that remaining in gland lysates ($p > 0.05$). However, the proportion of the Fc fragment detected in saliva increased slightly, but significantly, in response to the use of isoproterenol along with pilocarpine stimulation ($p < 0.01$; Figure 4A). The percentage of the total amount of the Fc fragment produced that was secreted was also calculated, individually for each animal, and there was no significant difference in this value between rats treated with pilocarpine $\pm$ isoproterenol ($p > 0.05$; Figure 4B). When AdhGH was administered into rat submandibular glands, essentially all hGH secreted was found in saliva, as previously reported [12,19] (not shown).

Is there an *in vivo* interaction between the Fc fragment and hGH after vector co-administration to rat submandibular glands?

Next, we aimed to determine whether simultaneous expression of transgenic hGH and the Fc fragment influenced each other's secretory behaviour from rat submandibular glands. In one set of experiments, rats received either 10^9 vp of AdFc or AdhGH in one submandibular gland. In another set of experiments, performed the next day on other animals from the same cohort, the rats received a mixture of 10^9 vp of both AdFc and AdhGH simultaneously in the same gland. Forty-eight hours after the delivery of each vector, combined pilocarpine and isoproterenol stimulation was used, as described above, to stimulate salivation.

As shown in Figure 5A, the percentage of the total amount of detectable Fc fragment that was secreted was slightly, but not significantly, influenced by separate or simultaneous vector delivery ($p > 0.05$; approximately 20.8%, median for separate delivery versus approximately 7.0% for simultaneous delivery). Similarly, the percentage of hGH secreted from transduced glands was comparable under both conditions (not shown). The serum-to-saliva distribution ratios of Fc in individual rats are shown in Figure 5B. As indicated, the median serum-to-saliva distribution ratio of the secreted Fc fragment was approximately 74 when AdFc was administered alone and approximately 134 when AdFc and AdhGH were administered together, and this difference was not significant ($p > 0.05$). Almost all secreted hGH (>99%) was found in saliva (not shown).

Immunolocalization of the Fc fragment and hGH after vector co-administration to rat submandibular glands

In transduced salivary epithelial cells, both the Fc fragment and hGH were detected in the cytoplasm with a punctate pattern (Figures 6A and 6B). Staining for hGH was localized over the nuclei, towards the apical pole of the cell, occupying most of the cytoplasm. Conversely, the immunofluorescent signal for the Fc fragment was localized primarily in the basal part of the cell. In epithelial cells that expressed both hGH and Fc protein together (Figures 6A and 6B), the immunofluorescent signal distribution was generally unchanged compared to that seen in other cells expressing either protein alone (not shown). However, in some cells, we observed a co-localization of the Fc fragment and hGH in the punctate cytoplasmic pattern (yellow colour in the merged image), although, generally, the immunofluorescent signals for hGH and the Fc fragment were distinct.

Discussion

We studied the secretion of the mouse IgG2b Fc fragment from rodent submandibular glands *in vivo*. Immunoglobulins, in particular the kappa light chain or heavy chain Fc fragment derivatives, often have been used as markers of constitutive secretion, and their sorting mechanisms have been extensively studied *in vitro* [15]. Specifically, in the present study, we

investigated: (i) how the secreted mouse Fc fragment is distributed between serum and saliva; (ii) whether the serum-to-saliva distribution is affected by the nature of stimulation of salivary secretion; and (iii) whether the secretion pattern of Fc fragment is altered by co-expression of transgenic hGH.

To study the sorting of Fc fragment, we created a first-generation, replication deficient, recombinant adenovirus encoding mouse immunoglobulin Fc fragment (AdFc). When AdFc was administered into rat submandibular glands, it resulted in the dose-dependent, predominantly basolateral, secretion of the Fc fragment following pilocarpine stimulation (Figure 2). Similarly, when AdFc was administered into mouse submandibular glands at a single dose of 10^9 vp/gland, the Fc fragment was again secreted primarily into the bloodstream (Figure 3).

We next examined the secretion of the transgenic Fc fragment when co-expressed with transgenic hGH, a RSP protein that physiologically is stored in granules of neuroendocrine cells and, as a transgenic protein, is primarily secreted into saliva from salivary glands *in vivo*, presumably via the RSP [1,19,20]. We found that the Fc fragment was secreted predominantly into the bloodstream from rat submandibular glands when it was expressed alone, or if co-expressed with transgenic hGH, and when salivary secretion was stimulated by pilocarpine $\pm$ isoproterenol (Figures 4 and 5).

The notion of salivary proteins being secreted into the bloodstream is well recognized [21], although the precise basolateral secretory pathways involved have not been characterized in salivary glands [5,6]. Previously, we demonstrated that basolateral secretion of transgenic secretory proteins from rodent salivary glands primarily occurred when constitutive pathway secretory proteins were encoded by the transgene (e.g. human α -1-antitrypsin [4] or hEPO [11,12]). Conversely, when the transgene encoded a RSP protein (e.g. hGH), basolateral (i.e. endocrine) secretion only occurred when the hGH was produced in excess [17,19]. Because human α -1-antitrypsin and hEPO are physiologically considered to be secreted by the constitutive secretory pathway [22,23], the results obtained in our study suggest that the mouse Fc fragment is secreted via a basolateral constitutive or constitutive-like pathway from rat submandibular glands.

All of the results obtained in the present study are consistent with this conclusion. Although we detected some increase in the secretion of the Fc fragment into saliva after stimulation with pilocarpine plus isoproterenol, compared to secretion in response to pilocarpine stimulation alone, the amount was small and consistent with the *in vitro* findings of Castle *et al.* [15] demonstrating that the Fc fragment can enter the RSP in AtT20 cells *in vitro*. Because the relative amount of Fc fragment in saliva is quite low, it appears that, in rodent salivary glands, either the Fc fragment enters secretory granules *in vivo* inefficiently or it is rapidly removed from the forming granules, as was the case *in vitro* in mouse pituitary AtT20 cells [15]. This conclusion is further supported by immunofluorescence imaging of the Fc fragment and hGH when they are co-expressed as transgenes in the same rat submandibular epithelial cells (i.e. very little merging of the separate signals is found) (Figure 6). This finding is thus consistent with a sorting-by-retention model within the RSP for the Fc fragment in the rat submandibular gland.

The mechanisms involved in sorting of non-RSP proteins are not well understood and, classically, have been assumed to occur by default [24]. However, they may be affected by binding to other cargo proteins or by being incorporated into aggregates of regulated secretory proteins [25]. For example, anti-chromogranin B antibody, a constitutive secretory protein, can be diverted to the regulated pathway by protein-protein interactions with chromogranin B, a RSP protein [26]. However, in the present study, neither the endogenous RSP proteins produced

by rat submandibular glands, nor the co-expression of transgenic hGH in this tissue, dramatically influenced the proportion of the Fc fragment secreted into saliva or serum.

In summary, we have described for the first time the *in vivo* sorting of a transgenic paradigm constitutive secretory protein, the mouse Fc fragment, in rodent submandibular salivary glands. This protein is secreted almost entirely into serum in both rats and mice, apparently via a constitutive or constitutive-like pathway.

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Figure 1.

First-generation serotype 5 recombinant adenovirus (Ad5) vector encoding the secreted mouse Fc fragment. The prerenin-Fc cDNA fragment was cloned into the pACCMV pLpA shuttle vector between the *EcoRI* and *HindIII* sites. The resulting pACCMVpreFc plasmid was cotransfected with the helper plasmid pJM17 into 293 cells to yield a recombinant Ad5 vector encoding secreted mouse Fc fragment (AdFc), which was then amplified and purified as described in the Materials and methods. 293 cells were transduced overnight with AdFc at 10 vp per cell, and the secreted Fc fragment was detected in the medium as a 30-kDa protein by immunoblotting with an anti-mouse secondary antibody as described in the Materials and

methods. Lanes from left to right: protein molecular weight standard, 2 μ l of conditioned medium, 12.5 μ l of conditioned medium. The molecular mass (kDa) is indicated to the left

Figure 2.

Dose-dependent production of the transgenic mouse Fc fragment by rat submandibular glands *in vivo*. 10^8 , 10^9 or 10^{10} vp of AdFc were administered into single submandibular glands of male Wistar rats. After 48 h, saliva was collected using 0.5 mg/kg of subcutaneous pilocarpine stimulation, then blood was collected and transduced glands harvested from euthanized animals. The Fc fragment concentration in each sample was determined with a mouse IgG2b ELISA Kit. The Fc fragment concentrations in serum, saliva and targeted gland extracts are shown as a function of vector dose. Data are the mean $\pm$ SEM of three animals in each dose group. Note that little Fc fragment was detected in saliva, even at the highest vector dose administered

Figure 3.

Secretion of the mouse Fc fragment from mouse submandibular glands. 10^9 vp of AdFc was administered into single submandibular glands of male BALB/c mice. After 48 h, 0.5 mg/kg of subcutaneous pilocarpine was used to induce salivation, and saliva and blood were collected. Samples of sera (0.2 μ l) and saliva (2 μ l), obtained from five treated mice, and, as a control, 0.2 μ l of serum from one nontreated mouse, were electrophoresed and immunoblotted with anti-mouse secondary antibodies as described in the Materials and methods. The molecular mass (kDa) is indicated on both sides of the figure. The Fc fragment was detected as an immunoreactive band of approximately 30 kDa (arrow)

Figure 4.

Effect of β -adrenergic stimulation on the secretion of the mouse Fc fragment into serum and saliva from rat submandibular glands *in vivo*. 10^9 vp of AdFc was administered to single submandibular glands of male Wistar rats. After 48 h, saliva was collected using 0.5 mg/kg of pilocarpine (PIL) stimulation, with or without 5 mg/kg of isoproterenol (IPR; a β -adrenergic agonist), then blood was collected and transduced glands harvested from euthanized animals. The Fc fragment concentration was determined with a mouse IgG2b ELISA Kit. (A) Presence of the Fc fragment in serum (ser) and saliva (sal) in response to different stimuli, expressed as percentage of total amount of transgenic protein produced (i.e. in saliva, serum, plus gland extracts). (B) Percentage of the secreted Fc fragment in response to different stimuli, secreted into both serum plus saliva. Median values are indicated by horizontal bars; * $p < 0.05$; ns, not significant. Each circle represents data obtained from an individual animal

Figure 5.

Secretion of the mouse transgenic Fc fragment after expression alone and when co-expressed with transgenic human growth hormone (hGH). 10^9 vp of AdFc were administered to single submandibular glands of male Wistar rats either alone or in combination with 10^9 vp of AdhGH. After 48 h, saliva was collected using stimulation with 0.5 mg/kg of pilocarpine and 5 mg/kg of isoproterenol, then blood was collected and transduced glands harvested from euthanized animals. The Fc fragment concentration was determined with a mouse IgG2b ELISA Kit. (A) Proportion of the secreted Fc fragment (i.e. amounts in saliva plus serum) either after separate expression or co-expression. (B) Serum-to-saliva distribution ratios of the transgenic Fc fragment expressed separately or in combination with transgenic hGH. Median values are indicated by horizontal bars; ns, not significant. Each circle represents data obtained from an individual animal

Figure 6.

Immunolocalization of the transgenic Fc fragment and hGH within rat submandibular epithelial cells. 10^9 vp each of AdFc and AdhGH were delivered to submandibular glands of male Wistar rats. Forty-eight hours after delivery, glands were removed and processed for immunofluorescence microscopy, and localization of hGH and Fc fragment was detected using a confocal microscope as described in the Materials and methods. (A, B) Two different acini showing the presence of the transgenic Fc fragment and hGH. DAPI-stained nuclei are shown in blue; red staining shows hGH; and green indicates the Fc fragment. Scale bars = 10 μ m