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A recombinant adenoviral vector encoding functional vasoactive intestinal peptide

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Abstract

Vasoactive intestinal peptide (VIP) is a small neuropeptide, which exerts pleiotropic functions. Based on its immunomodulatory, secretory, and possibly trophic effects, VIP is a valuable candidate molecule for the management of autoimmune disease. The purpose of this study was to develop a recombinant viral vector capable of directing the expression of functional VIP. The vector rAd5CMVhVIP was constructed and used to infect 293 cells. VIP expression was measured by an ELISA and function was evaluated by measurement of intracellular cAMP formation. rAd5CMVhVIP directed VIP expression and the transgenic VIP elicited a dose-dependent increase of intracellular cAMP, mediated through the VIP receptor $VPAC_1$. This is the first report showing the construction of a recombinant viral vector encoding biologically active VIP.

Keywords: Vasoactive intestinal peptide; Gene transfer; Adenovirus; Autoimmune disease

Vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide. Originally thought to be solely a gastrointestinal peptide, it is widely distributed and has pleiotropic functions in the digestive tract, central nervous system, cardiovascular circuit, respiratory tract, endocrine glands, immune milieu, and metabolic processes [1]. Based on its immunomodulatory properties, VIP may be useful in the management of several autoimmune disorders [2]. For example, in a mouse model for rheumatoid arthritis, systemic administration of VIP led to clinical improvement and a decrease in pro-inflammatory, and an increase in anti-inflammatory, mediators [3]. In addition, VIP is a non-cholinergic, non-adrenergic neurotransmitter with secretory and possibly trophic effects [4,5].

The stability, and therefore the bioavailability, of exogenous VIP is limited due to its rapid degradation and inactivation [6]. Gene transfer potentially offers a means of sustained expression of a VIP transgene. Since high serum VIP levels are associated with secretory diarrhea in patients with a VIPoma [7], local therapy of VIP is preferable. For example, salivary glands provide an excellent target site for localized gene transfer following retrograde ductal infusion of vectors [8]. Sjögren's syndrome (SS) is an autoimmune exocrinopathy of unknown etiology, mainly affecting the salivary and lacrimal glands, and could potentially benefit from VIP gene therapeutic treatment. The purpose of this study was to develop a recombinant vector encoding human VIP, as well as to examine VIP expression and biofunctionality in vitro.

Materials and methods

Construction of viral vector encoding functional VIP. VIP cDNA was obtained by RT-PCR of female fetal human brain mRNA (Stratagene, La Jolla, CA, USA) with specific VIP primers (Fig. 1). The specific oligonucleotide primers, containing *Not*I sites (underlined), used were: sense primer 5′-AAGCGGCCGCCCTAAGACAGCTC

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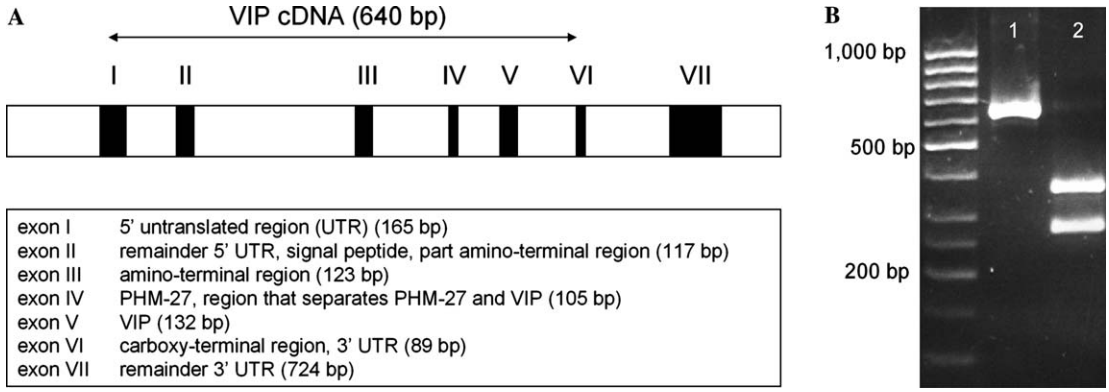

Fig. 1. Generation of VIP cDNA by RT-PCR. (A) Organization of the human VIP/PHM-27 gene: seven exons (black boxes) and six introns (white boxes). VIP cDNA, obtained by RT-PCR of female fetal human brain mRNA, was derived from exons I–VI (arrow). (B) Lane 1 shows the PCR product, corresponding to the VIP cDNA (640 bp). Lane 2 shows the VIP cDNA after digestion with *Hin*cII, yielding expected 365 and 275 bp cDNA fragments. Samples were run on a 3% agarose gel with a DNA ladder (left side). VIP cDNA was also verified by sequencing.

CAAAACAAACC-3′ and anti-sense primer 5′-ATAGCGGCCGC GGGAAGTTGTCATCAGCTTTGC-3′. The PCR, using *PfuTurbo* DNA polymerase (Stratagene), was performed as follows: denaturing at 95 °C for 30 s, extension at 68 °C for 30 s, and annealing at 74 °C for 1 min for 35 cycles, followed by 72 °C for 10 min.

The PCR-amplified VIP cDNA (640 bp) was verified by restriction digests (Fig. 1) and by sequencing (not shown). The cDNA was digested with *Not*I and inserted into the pAC shuttle plasmid [9] to yield pAC-CMV-hVIP (size 9440 bp). The serotype 5 adenoviral vector, rAd5CMVhVIP, was generated by homologous recombination after cotransfection with pJM17 in 293 (Microbix; see below) cells as described [9]. The infectious titer was determined by limiting dilution plaque assay and the particle titer by real-time quantitative PCR (Q-PCR; Applied Biosystems, Foster City, CA, USA) using specific VIP Q-PCR primers: sense primer 5′-CCAGCTCCTTGTGCTCC TG-3′ and anti-sense primer 5′-TGTAAAGAGGCCATGCCGA-3′.

Reagents and cell lines. The cell lines used were human embryonic 293 cells (ATCC, Manassas, VA, USA), 293 Microbix cells (Microbix Biosystems, Toronto, Canada), and a murine pituitary tumor cell line (AtT20 cells; a generous gift of Dr. N.X. Cawley, NICHD, NIH). 293 cells were grown in improved minimum essential medium, Eagle's (IMEM) and AtT20 cells in high-glucose Dulbecco's modified Eagle's medium (DMEM). All media were supplemented with 10% 55 °C heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD, USA), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL) (Biofluids, Rockville, MD, USA).

VIP measurement assay. Recombinant vector was used to infect cells. VIP expression and secretion were measured by an ELISA (Peninsula Laboratories, San Carlos, CA, USA) of culture media and cell lysates. The assay sensitivity was ~0.05 ng/mL.

cAMP assay. Intracellular cAMP formation was measured after incubating naïve 293 cells, which possess $VPAC_1$-receptors [10], in conditioned media obtained from 293 cells previously infected with rAd5CMVhVIP at a multiplicity of infection (MOI) of 320. Transgenic VIP in media was concentrated with dialyzers (Sigma, St. Louis, MO, USA, catalog # Z36,841-5) and diluted with non-conditioned media to yield the VIP concentrations shown in Fig. 3. Concentrated media from uninfected cells were used as a control and diluted as described above. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 10^{-4} M; Sigma) was added to minimize cAMP degradation. Intracellular cAMP formation was determined with a cAMP (low pH) ELISA (R&D Systems, Minneapolis, MN, USA). The lower detection limit was ~0.39 pmol/mL. To block the $VPAC_1$ receptor, the selective $VPAC_1$-receptor antagonist ([Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{17}]VIP(3–7)/GRF(8–27)) [11], at either 30 or 300 nM, was incubated with concentrated conditioned or unconditioned media and cAMP assays, as above, were performed.

Results and discussion

The infectious titer of the rAd5CMVhVIP vector was 7.0×10^{11} pfu/mL and the physical titer 6.5×10^{12} particles/mL. To determine if this vector directed VIP protein expression, both 293 and AtT20 cells were infected. Typically, 3.2×10^2 particles of rAd5CMVhVIP/cell resulted in ~3.3 ng VIP (32-fold increase over background) in the culture medium per 10^6 infected 293 cells (Fig. 2). Similar results were found in an experiment conducted with AtT20 cells (3.4 ng/10^6 cells in 24 h, MOI 320). Transfected cell lysates did not result in measurable VIP levels (data not shown), indicating all produced VIP was secreted by the cells.

The biological function of the transgenic VIP secreted into the culture media was evaluated by measuring intracellular cAMP formation after incubating naive 293 cells in conditioned media obtained from 293 cells previously infected with vector. Transgenic VIP elicited a dose-dependent increase in cAMP at concentrations from 10^{-12} to 10^{-8} M with maximal increases in cAMP levels achieving ~5–10-fold of the basal level (IBMX alone; Fig. 3). This corresponded to a value of ~15 pmol cAMP per 10^6 cells. Next, we used a selective $VPAC_1$-antagonist to determine if transgenic VIP elicited cAMP formation in a receptor-specific manner. The antagonist used, [Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{17}]VIP(3–7)/GRF(8–27), resulted in a dose-related decrease in cAMP formation elicited by transgenic VIP (Fig. 4). At 300 nM antagonist, transgenic VIP-induced cAMP elevations were blocked by more than 80%.

Recombinant VIP protein leads to clinical improvement in a mouse model for rheumatoid arthritis [3,12]. This result suggested the possible advantageous effects

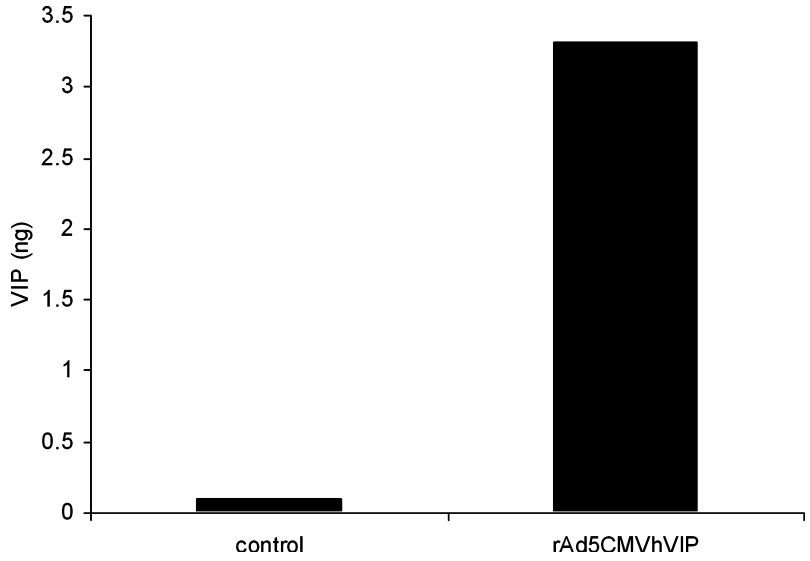

Fig. 2. VIP expression in rAd5CMVhVIP-infected cells. Culture media were assayed for VIP by an ELISA as described in Materials and methods. Data shown are the total amount of VIP (ng) per 10^6 infected 293 cells (MOI 320) per 24 h. The results are representative of five experiments with 2–4 replicates.

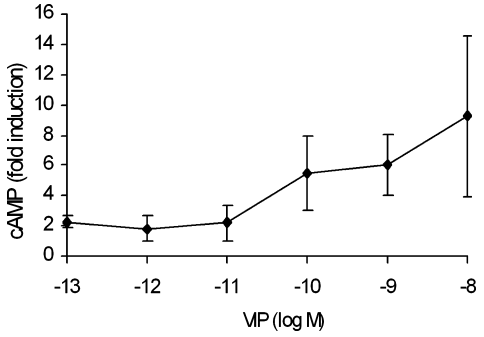

Fig. 3. Transgenic VIP stimulation of intracellular cAMP formation. Naïve 293 cells were incubated in concentrated media obtained from 293 cells previously infected with rAd5CMVhVIP (MOI of 320) or concentrated control media from uninfected cells. Intracellular cAMP formation was determined as described in Materials and methods. The data are expressed as fold stimulation of cAMP levels compared to basal levels and are means ± SEM ($n = 3$). Each experiment was assayed in duplicate.

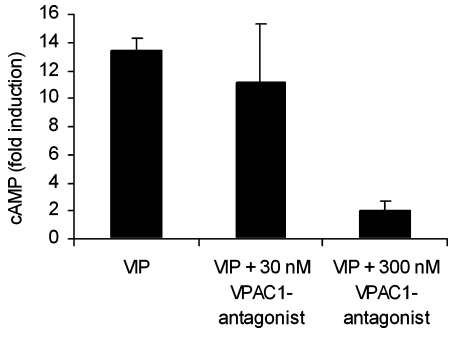

Fig. 4. Effect of a $VPAC_1$ antagonist on intracellular cAMP formation elicited by transgenic VIP. Naïve 293 cells were incubated with conditioned media from VIP vector infected cells (VIP 10^{-8} M, obtained as in Fig. 3) and the selective $VPAC_1$-receptor antagonist ([Ac-His[1], D-Phe[2], K[15], R[16], L[17]]VIP(3–7)/GRF(8–27)) at either 30 and 300 nM. Intracellular cAMP formation was determined as described in Materials and methods. The data are expressed as fold stimulation of cAMP levels compared to basal levels and are means ± SEM ($n = 2$). Each experiment was assayed in duplicate.

of VIP in other autoimmune diseases [2,3,13]. To our knowledge this is the first report of the development of a gene transfer vector encoding functional VIP for such potential therapeutic use. Indeed, we are aware of only one report about the expression of the human VIP gene in transgenic mice [14]. The likely reason for this minimal study is the complex nature of the VIP gene and the extensive processing that is required prior to the generation of functional VIP protein (see below). In the present study, we constructed a recombinant virus rAd5CMVhVIP to examine the in vitro expression and function of the VIP transgene product.

VIP, together with pituitary adenylate cyclase-activating polypeptide (PACAP) and peptide histidine–methionine (PHM-27), is a member of the glucagon/secretin superfamily [15]. The precursor protein, prepro-VIP/PHM-27, is encoded on human chromosome 6 [16] and the 8837 bp spanning gene contains seven exons and six introns [17] (Fig. 1). Extensive post-translational processing of the prepro-protein takes place during formation and maturation of secretory granules [18,19]. Additionally, as with many neuropeptides, amidation of the C-terminal amino acid (in the case of VIP glycine in the C-terminal sequence) is important for biological activity [20,21]. Although functional VIP generation typically requires extensive post-translation processing, Simoncsits et al. showed that elongated precursors of

human VIP that have not undergone α-amidation were functionally active [22]. In addition, biologically active VIP with a C-terminal extension of glycine–lysine–arginine has been isolated from porcine intestinal extracts [23]. This suggests that in tissues or cells lacking amidation enzymes it still may be possible to generate functional VIP.

The neuro-endocrine cell line AtT20 contains secretory granules and key enzymes needed for VIP processing [20,24,25]. While 293 cells lack regulated secretory granules and at least some key processing enzymes [25], when both cell types were infected with rAd5CMVhVIP, we observed similar levels of VIP expression. Since 293 cells have VIP receptors, we chose to perform additional studies with these cells. Transgenic VIP elicited a dose-dependent increase of intracellular cAMP formation in 293 cells, an effect mediated through the specific VIP cell surface receptor, $VPAC_1$, as shown by inhibition of cAMP increases by the $VPAC_1$-antagonist [Ac-His^1, D-Phe^2, K^{15}, R^{16}, L^{17}]VIP(3–7)/GRF(8–27). VIP in the immune system can act as an autocrine regulator [26]. These effects, mainly mediated through the $VPAC_1$-receptor, are wide ranging; including the inhibition of several macrophage functions, T cell proliferation, lymphocyte migration, and the expression of chemokines and pro-inflammatory cytokines [13,26,27].

In conclusion, this is the first report of the development of a recombinant viral vector capable of directing biologically functional transgenic VIP. As such, this vector likely will be useful for numerous investigators interested in studying VIP biology in diverse model systems. Whether the vector will be useful for local management of autoimmune disease, however, remains to be established.

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