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ACCELERATED COMMUNICATION

Identification of a Human Gastrointestinal Tract and Immune System Receptor for the Peptide Neuromedin U

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ABSTRACT

Neuromedin U (NmU) is a 25 amino acid peptide prominently expressed in the upper gastrointestinal (GI) tract and central nervous system. It is highly conserved throughout evolution and induces smooth muscle contraction in a variety of species. Our understanding of NmU biology has been limited because the identity of its receptor was unknown. Here we demonstrate that GPR66/FM-3 is specifically stimulated by NmU, causing the mobilization of intracellular calcium. This response was dose-dependent (EC_{50} = 10 nM) and specific in that none of over 1000 ligands tested, including other neuromedins (NmB, C, L, K, N), induced a calcium flux in GPR66/FM-3-transfected cells. The GPR66/FM-3 mRNA is prominently expressed in the upper GI tract of humans, as is the mRNA for NmU, consistent with role for this receptor-ligand pair in regulating the function of this organ system. In addition, we show that whereas neuromedin U is expressed by monocytes and dendritic cells, GPR66/FM-3 is expressed by T cells and NK cells. These data suggest a previously unrecognized role for NmU as an immunoregulatory molecule.

The neuromedins (Nm) are a group of smooth muscle-stimulating peptides commonly divided into four groups: bombesin-like (NmB, NmC), kassinin-like (NmL/neurokinin A, NmK/neurokinin B), neurotensin-like (NmN), and neuromedins U (NmU). Among this group of peptides, neuromedin U is the least well understood, in large part due to the lack of a known receptor. Neuromedin U was first reported by Minamino et al. (1985a,b) as a peptide isolated from porcine spinal cord. These investigators isolated two active peptides, NmU-25 and a further cleavage product, NmU-8, and characterized them as having smooth muscle contractile activity. Neuromedin U was subsequently isolated from a variety of species including rat (Conlon et al., 1988; Minamino et al., 1988), guinea pig (Murphy et al., 1990), dog (O'Harte et al., 1991), rabbit (Kage et al., 1991), chicken (Kage et al., 1991; Domin et al., 1992), and frog (Salmon et al., 2000).

The cDNA for rat NmU was the first to be cloned and analysis of the nucleic acid sequence suggests that NmU is produced as a 174 amino acid precursor. The precursor contains a signal peptide and several dibasic cleavage sites that give rise to a number of possible secreted peptides, including NmU, which is present near the carboxyl-terminus (Lo et al., 1992). The human NmU cDNA was subsequently cloned and encodes a similar 174 amino acid precursor (Austin et al., 1995). Neuromedin U shows remarkable conservation throughout evolution and a core active peptide (Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2) is absolutely conserved among mammalian species (Fig. 1).

A variety of biological activities have been reported for NmU although its role in normal physiology is unclear. The first biological activity ascribed to NmU was smooth muscle contraction (Minamino et al., 1985a,b). These experiments have not been consistent among different species, however, in regard to the specific tissues that respond to NmU. For example, NmU has been reported to stimulate the contraction of turtle small intestine (Bockman et al., 1989), human ileum and urinary bladder cells (Maggi et al., 1990), and rat stomach circular muscle (Benito-Orfila et al., 1991). In contrast, no NmU-induced contractile response was observed in guinea pig small intestine (Minamino et al., 1985a), porcine jejunum (Brown and Quito, 1988), or frog intestine (Benito-Orfila et al., 1991). Neuromedin U has also been reported to

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ABBREVIATIONS: Nm, neuromedin; GI, gastrointestinal; GPCR, G protein-coupled receptor; TM, transmembrane; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; bp, base pair(s).

increase arterial blood pressure (Sumi et al., 1987; Gardiner et al., 1990) and modify ion transport in the intestinal tract (Brown and Quito, 1988). Finally, NmU injected s.c. into rats has been reported to increase circulating ACTH levels in the short term and to increase serum corticosterone levels in the long term (Malendowicz et al., 1993, 1994), suggesting a role in regulation of the hypothalamo-pituitary-adrenal axis.

The orphan G protein-coupled receptor (GPCR) GPR66/FM-3 was originally identified as a partial mouse expressed sequence tag residing in GenBank. A full-length mouse cDNA was cloned from a T cell library and subsequently used as a probe to identify a human clone (Tan et al., 1998). A BLAST comparison of the human cDNA sequence of GPR66/FM-3 to public DNA databases revealed a genomic clone (AC017104) that contained the entire open reading frame of GPR66/FM-3. Although the gene structure for GPR66/FM-3 was previously described (Tan et al., 1998), the sequence was not reported. The open reading frame for GPR66/FM-3 is encoded on two exons. The first exon encodes the putative start methionine (in an appropriate Kozak context with an upstream, in-frame stop codon) through transmembrane (TM) domain six. The second exon encodes the third extracellular loop, TM domain seven and the carboxyl-terminus. Comparison of GPR66/FM-3 to other known GPCRs shows it is most similar to the human growth hormone secretagogue and neurotensin receptors (33 and 29% amino acid identity, respectively) as well as the recently described motilin receptor (Feighner et al., 1999). Phylogenetic analysis, however, indicates that this receptor lies on a distinct evolutionary branch (not shown).

We have developed and employed a "reverse pharmacology" approach (a term referring to the process by which an orphan G protein-coupled receptor is identified and used as "bait" to identify its natural ligand) to identify the natural ligand for GPR66/FM-3. Similar approaches have recently proven successful in identifying ligands for several previously orphan receptors (Chambers et al., 1999, 2000; Feighner et al., 1999), and we report here the identification of neuromedin U as a ligand for GPR66/FM-3.

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hNmU  --MLRTESCRPRSPAGQVAAASPLLLLLLLLAWCAGACRGAPILPQGLQPEQQLQLWNEI
rNmU  MSRAANRRPGLSAGQLAAATASPLLSLLLLLACCADACRGTPISPQRLPPEQELQLWNEI
mNmU  MSRAAGHRPGLSAGQLAAATASPLLSLLLLLACCADACKGVPISPQRLQPEQELQLWNEI

hNmU  DDTCSSFLSIDSQPQASNALEELCFMIMGMLPKPQEQDEKDNTKRFLFHYSKTQKLGKSN
rNmU  PEACASFLSVDSQPQASVALRKLCRVLMEIFQKPQEQTEKDNAKRFLFHYSKTQKLGNSN
mNmU  HEACASFLSIDSRPQASVALRELCRIVMEISQKPQEQSEKDNTKRFLFHYSKTQKLGNSN

hNmU  VVSSVVHPLLQLVPHLHERRMKRFRVDEEFQSPFASQSRGYFLFRPRNGRRSAGFI
rNmU  VVSSVVHPLLQLVPQLHERRMKRYKVN-EYQGP-VAPSGGFFLFRPRNGKRSTSFI
mNmU  VVSSVVHPLLQLVPQLHERRMKRFKA--EYQSPSVGQSKGYFLFRPRNGKRSTSFI
pNmU  ----------------------FKVDEEFQGPIVSQNRRYFLFRPRN--------
dNmU  ----------------------FRLDEEFQGPIASQVRRQFLFRPRN--------
rbNmU ----------------------FPVDEEFQSPFGSRSRGYFLFRPRN--------
gpNmU ------------------------------------GYFLFRPRN--------
cNmU  ----------------------YKVDEDLQGAGGIQSRGYFFFRPRN--------
fNmU  ----------------------LKPDEELQGPGGVLSRGYFVFRPRN--------
tfNmU ----------------------SDEEVQVPGGVISNGYFLFRPRN--------
                                          * *****
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Fig. 1. Alignment of neuromedin U from various species demonstrates the conservation of a core active peptide. The amino acid sequences of various NmUs were obtained from GenBank and aligned using CLUSTAL W. Human (h), rat (r), and mouse (m) NmU sequences are predicted from their respective cDNAs, whereas pig (p), dog (d), rabbit (rb), guinea pig (gp), chicken (c), common frog (f), and tree frog (tf) NmU sequences were obtained from the GenBank peptide database and were originally derived isolated peptides. Sites of potential dibasic cleavage are in bold and the conserved core peptide is boxed. Absolutely conserved residues within the core sequence are noted with an asterisk.

Materials and Methods

Cloning and Expression of GPR66/FM-3. The coding region of GPR66/FM-3 was amplified from human spleen cDNA by polymerase chain reaction (PCR) using primers specific for GPR66/FM-3 [forward primer containing a consensus Kozak sequence (underlined): 5′-GCCGCCACCATGGCTTGCAATGGCAGTGCGGC-3′; reverse primer 5′-TCAGGATGGATCGGTCTCTTGCTG-3′]. Thermocycling conditions included a hot start at 94°C for 1 min, followed by 35 cycles of 94°C, 30 s; 60°C, 30 s; 72°C for 1 min 30 s; and a final extension at 72°C for 7 min. The resulting PCR product was cloned into pCR3.1 (Invitrogen, Carlsbad, CA) and the insert then sequenced using an ABI Prism dRhodamine dye DNA sequencing kit and ABI 377 automated sequencer (PE-Biosystems, Foster City, CA). Transient and stable expression of GPR66/FM-3 in HEK 293 cells was accomplished using LipofectAMINE 2000 (GibcoBRL Life Technologies, Gaithersburg, MD). Briefly, 24 h before transfection HEK 293 cells were seeded into 75 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), then transfected overnight using LipofectAMINE 2000. The following day cells were removed from the plate (0.05% trypsin, 0.5% EDTA), washed, and either replated for screening as detailed below or placed under selection in DMEM containing 10% FCS and G418 (GibcoBRL) at a concentration of 1 mg/ml. Cells surviving initial selection (2 weeks) were subsequently maintained in DMEM/10% FCS containing G418 at 0.5 mg/ml.

Ligand Screening. All initial screening was accomplished using transiently transfected cells generated as described in the preceding section. Twenty-four hours before screening, transfected cells were replated into clear bottom, black-walled 96-well plates precoated with poly (D-lysine) (Becton-Dickinson, Franklin Lakes, NJ) at a density of 5×10^5 cells/well. The day of screening (48 h post-transfection) cells were loaded for 1 h with Fluo-3 AM (Sigma Chemical Corp., St. Louis, MO) at a concentration of 2 mM in DMEM/10% FCS containing 2.5 mM probenecid (Sigma) and 20 mM HEPES buffer solution (pH 7.55, GibcoBRL). Cells were then washed 4× in Hanks'

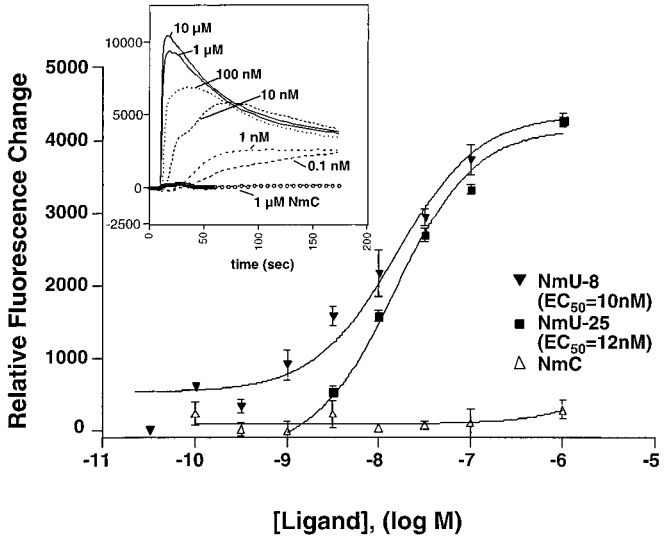
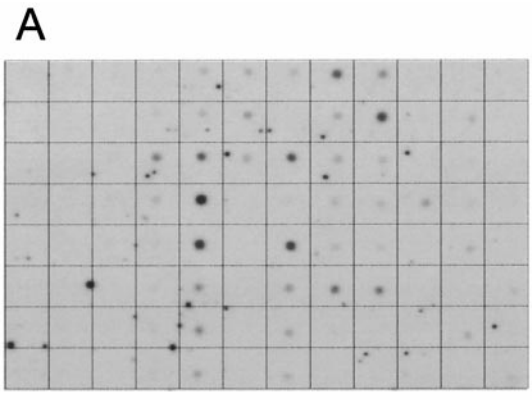


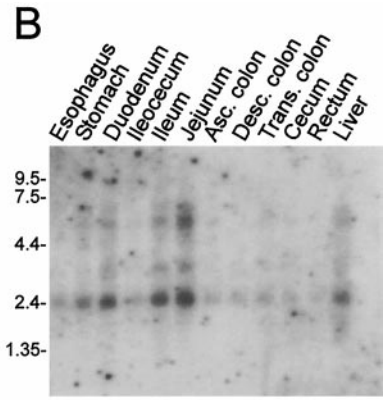
Fig. 2. GPR66/FM-3 expressing cells show a specific, dose-dependent response to neuromedin U. HEK 293 cells were transiently transfected with the GPR66/FM-3 cDNA and screened against a large collection of GPCR ligands. Mock transfected cells stimulated with NmU-25 and GPR66/FM-3 transfected cells stimulated with neuromedin C were used as controls. Results are plotted as change in fluorescence (peak height) versus log molar concentration. The EC_{50} for NmU-25 and NmU-8 is indicated, and each point is the average of four replicate samples. Inset, the dose-dependent intracellular calcium flux observed in response to human NmU-25 (10 μM-0.1 nM) is plotted as fluorescence counts versus time (s). HEK 293 cells stably expressing GPR66/FM-3 were used, and the response to neuromedin C is shown as a negative control.

buffered saline solution containing 2.5 mM probenecid, 20 mM HEPES, and 1% bovine serum albumin (wash buffer). Compounds used in screening were initially suspended in dimethyl sulfoxide (small molecules) or water (peptides) and diluted into wash buffer just before screening. Final dilutions resulted in a compound screening concentration of 3 μg/ml and contained less than 1% dimethyl sulfoxide, a concentration that generates no background signal in the screening assay used (data not shown). Screening was accomplished using the fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA), which allows real-time monitoring of fluorescence of all 96 wells simultaneously (Coward et al., 1999, Sullivan et al., 1999). The results obtained were expressed as a change in relative fluorescence units versus time or as relative fluorescence change versus concentration of ligand (log M).

Peptide ligands and small molecules used in the large scale screening of GPR66/FM-3 were obtained from a wide variety of commercial sources. Included in the ligand libraries was the LOPAC small molecule library (RBI, Natick, MA), which includes 640 compounds of various pharmacologic classes including adenosines, purinergics, adrenergics, histaminergics, cholinergics, ion channel modulators, dopaminergics, glutaminergics, opioids, serotonergics, and GABAergics (exact content can be obtained from manufacturer). The libraries also included well over 500 peptides that are known or suspected GPCR ligands. These were obtained from various commercial sources (RBI; Bachem, King of Prussia, PA; Sigma) or were custom synthesized. In particular, rat NmU-23, pig NmU-25, pig NmU-8, neuromedins B and C (bombesin-like), neurokinins A and B (the kassinin-like tachykinins, also known as neuromedins L and K, respectively), and neuromedin N (neurotensin-like) were obtained from Bachem, whereas HPLC-purified human NmU-25 and nonamidated NmU-8 were custom synthesized (Research Genetics, Huntsville, AL). Finally a commercial library of over 250 bioactive lipids including eicosanoids, octadecanoids, platelet-activating factors, and sphinoglipids was also tested (BioMol, Plymouth Meeting, PA).

Messenger RNA Expression Analysis. Expression of NmU and GPR66/FM-3 was examined using dot blots and northern blots obtained from a commercial source (CLONTECH, Palo Alto, CA). Hybridization to blots was carried out using PCR-generated DNA fragments encompassing 1200 bp of the coding region of GPR66/FM-3 or 400 bp of the NmU gene, beginning at the 3′-end and including most of the coding region. The DNA fragments were random-prime-labeled with [^{32}P]dCTP, and the blots hybridized for 14 h in ExpressHyb (CLONTECH) containing 2×10^6 cpm/ml of radiolabeled probe. The following day the blots were washed and exposed to Kodak Biomax MS film for 3 days at 70°C. In addition to the dot blots, cDNA libraries prepared from various tissues and clonal cell lines were assayed for GPR66/FM-3 expression using real-time quantitative PCR. Briefly, 5 μg of total RNA was reverse tran-





	CNS			CV	GI		Immune, GU, misc.				Fetal	(-)
whole brain	cb left	subst. nigra	heart	esoph-agus	colon, trans	kidney	lung	liver	leukemia HL-60	fetal brain	yeast total RNA
cerebral cortex	cb right	accum-bens nucleus	aorta	stomach	colon, descend	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA 100 ng
frontal lobe	corpus callosum	thalamus	atrium left	duo-denum	rectum	spleen	bladder	adrenal gland	leukemia K-562	fetal kidney	E.coli rRNA 100 ng
parietal lobe	amyg-dala	pituitary gland	atrium right	jejunum		thymus	uterus	thyroid gland	leukemia MOLT-4	fetal liver	E. coli DNA 100 ng
occipital lobe	caudate n.	spinal cord	ventricle left	ileum		peripher leuko-cyte	prostate	salivary gland	Burkitt's lymphom Raji	fetal spleen	Poly r(A) 100 ng
temporal lobe	hippo-campus		ventricle right	ileo-cecum		lymph node	testis	mam-mary gland	Burkitt's lymphom Daudi	fetal thymus	human Cot1 DNA
para-central gray	medulla		heart septum	appen-dix		bone marrow	ovary		colorecta carcin. SW480	fetal lung	human DNA 100 ng
pons	putamen		heart apex	colon, ascend		trachea			lung carcin. A549		human DNA 500 ng

Fig. 3. Neuromedin U and GPR66/FM-3 have overlapping patterns of tissue expression. A, dot blot analysis of human GPR66/Fm-3 (top panel) and human NmU expression (middle panel) was performed as described under *Materials and Methods*. The tissues present on the blots and their positions on the blot are keyed (bottom panel). B, Northern blot analysis of human GPR66/FM-3 expression in digestive tract and related organs. Size markers are indicated.

scribed, and 20 ng of the resulting cDNA were analyzed for the expression of human GPR66/FM-3 by the SYBR Green PCR assay using a Perkin-Elmer GeneAmp 5700 Sequence Detection System (PE-Biosystems). A separate set of identical cDNAs was analyzed for the expression of hypoxanthine phosphoribosyltransferase (hprt) as an internal control and for quantification of the total amount of cDNA. For the SYBR Green assay, the following primer sets were used: GPR66/FM-3, 5-GCACGCCTACCAACTACTACC-3 (forward primer), 5-GCAGACCATCTCAAACAGTAGC-3 (reverse primer); human NmU, 5-TGGGAATGCTACCAAAGCCT-3 (forward primer), 5-TCATGCAGGTGAGGAACGAG-3 (reverse primer). Before running the cDNA libraries, the primer concentrations were optimized according to the manufacturer's specifications (Perkin-Elmer). The SYBR Green PCR reactions were carried out in 96-well plates containing the cDNA libraries as templates. In addition, the following components were added to a final reaction volume of 50 μl (all molarities are given as final concentrations): 10× SYBR Green buffer, 5.5 mM $MgCl_2$, 500 μM dNTP mixture, 1.25 U AmpliTaq Gold, 0.5 U AmpErase UNG, 200 μM each primer, and diethyl pyrocarbonate-treated water. The PCR reactions were carried out according to preset conditions for the GeneAmp 5700. The quantification of the amplicons in each well was determined according to the comparative Ct (threshold cycle number) method (PE Applied Biosystems, User Bulletin 2, 1997). Briefly, for each sample well, the formula used is $2E^{-(Ct_{target} - Ct_{standard})}$. This yields a quantification of the target (GPR66/FM-3 or NmU) PCR products in the experimental wells relative to the PCR products for the internal calibration (hprt) primers. These results were then plotted on a log scale. Any value of $\leq 10^{-5}$ required 35 or more cycles of amplification to visualize the product.

Results and Discussion

Unless a given receptor bears obvious homology to a known receptor, it is very difficult to predict even the nature (small molecule, peptide, protein, lipid, etc.) of the ligand that might interact with that receptor. To identify a natural ligand for GPR66/FM-3, a large and comprehensive collection of known and putative GPCR ligands was assembled from various sources, and this collection was then used in a calcium flux-based functional screen. This approach has been made tractable by the recent development of high-throughput assay systems such as the FLIPR.

Human embryonic kidney cells (HEK 293) were transiently transfected with GPR66/FM-3 cDNA and then assessed for their ability to flux calcium when stimulated with each of over 1000 compounds that are known or suspected to be GPCR ligands. These included small molecules, lipids, short peptides, and proteins. From among this library of potential ligands, only NmU generated a specific, dose-dependent calcium flux in the transfected cells (Fig. 2, inset). This response was dose-dependent with an EC_{50} of 10 nM and a maximal response observed between 1 and 10 μM (Fig. 2). Consistent with previous reports regarding the contractile activity of NmU (Minamino et al., 1985a,b; Brown and Quito, 1988; Bockman et al., 1989; Maggi et al., 1990; Benito-Orfila et al., 1991), we observed no significant difference in the ability of NmU-25/23 to stimulate GPR66/FM-3 when compared with NmU-8 (Fig. 2), which contains only the core

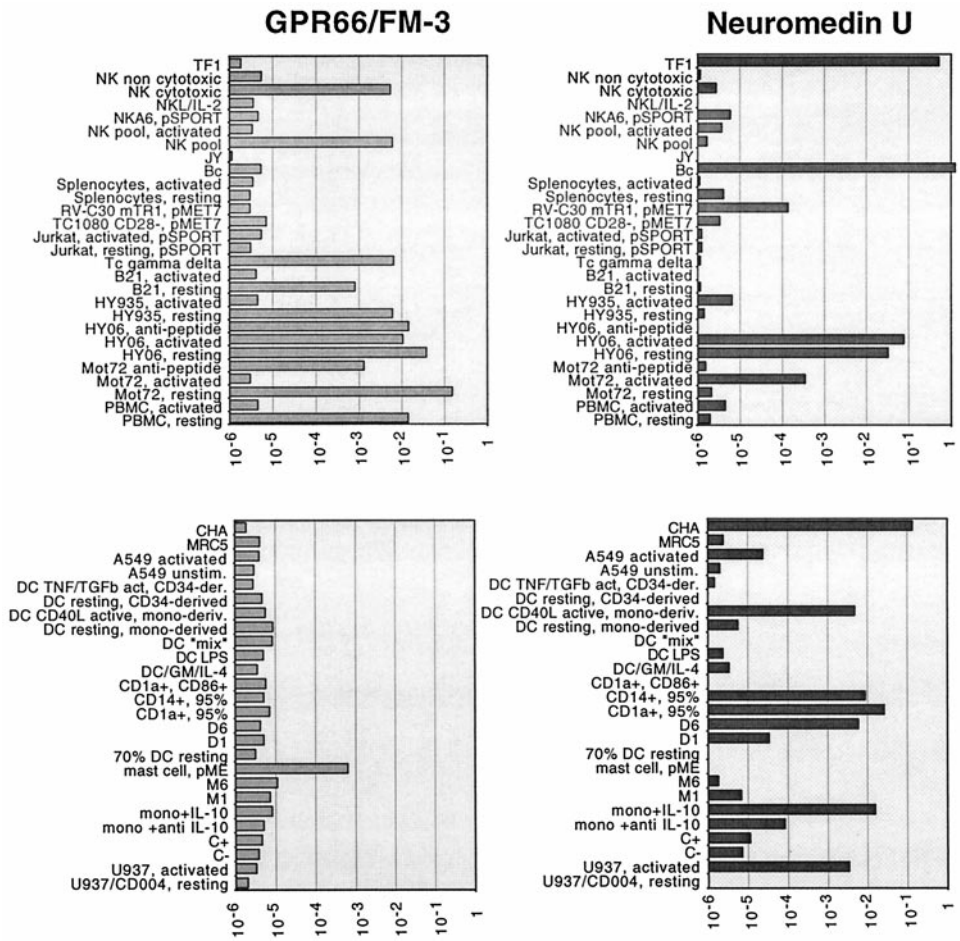


Fig. 4. Detailed analysis by quantitative PCR demonstrates distinct patterns of GPR66/FM-3 and NmU expression in lymphoid cells. The expression of GPR66/FM-3 and NmU in the immune system was examined in greater detail using quantitative PCR on cDNA libraries constructed from various lymphoid cell and tissue types. Results are displayed as a function of the ratio of target product to internal control product with anything above 10^{-5} considered significant (detailed under *Materials and Methods*). NK cells: NK (cytotoxic and non-cytotoxic) NK/IL-2, NKA6, NK pool; B cells: Jy, Bc, splenocytes; T cells: RV-C30, TC1080, Jurkat, Tc gamma delta, B21, HY935, HY06, Mot72; dendritic cells, CD34+ cell derived: DC TNF/TGFb, DC, DC CD40L activated; monocyte derived: DC resting, DC mix, DC LPS, DC GM/IL-4, CD1a+CD86+, CD14+, CD1a+, D6, D1, 70% DC; monocytes: M6, M1, mono+IL-10, mono+anti-IL-10, C+, C−, U937, U937/CD004 (all others are various cell lines and are given their common designation).

active domain. We did find amidation of the core peptide to be necessary for activity. Nonamidated NmU-8 did not activate the receptor even at concentrations in excess of 10 μM (data not shown). Other neuromedins including NmB and C (bombesin-like), NmL and K (also known as neurokinin A and B, the kassinin-like tachykinins), and NmN (neurotensin-like) were inactive.

To understand the possible physiologic role of the interaction of NmU with GPR66/FM-3, the expression of this ligand-receptor pair was comprehensively assessed using dot blots, Northern blots, and quantitative PCR. Neuromedin U mRNA was very broadly expressed and present in most tissues to some degree (Fig. 3A, middle panel). Expression was generally highest, however, in tissues of the upper gastrointestinal tract, including small intestine and stomach, and in certain cell lines (particularly the K562 and HL-60 erythroleukemia lines). The expression of GPR66/FM-3 as determined by dot blot was more restricted (Fig. 3A, top panel), but did overlap with the expression of its ligand (Fig. 1). As with NmU, GPR66/FM-3 expression was detected in tissues of the upper GI tract including stomach, duodenum, jejunum, and ileum. In addition, pancreas was also found to have high levels of GPR66/FM-3 and, somewhat surprisingly, GPR66/FM-3 was highly expressed in peripheral blood leukocytes and spleen. Aside from the expression in the upper GI tract and immune tissues, moderate expression of GPR66/FM-3 mRNA was also observed in placenta, heart, lung, mammary gland, and testis (Fig. 3A). Notably, little or no expression of GPR66/FM-3 was observed in nervous system tissues (Fig. 3A, top panel) although NmU is expressed in these tissues (Fig. 3A, middle panel) (Domin et al., 1987; Honzawa et al., 1987; Augood et al., 1988; Ballesta et al., 1988).

The expression of GPR66/FM-3 in the GI tract was also examined by Northern blot (Fig. 3B). The analysis confirmed the relative levels of expression of GPR66/FM-3 in the upper GI tract, with ileum and jejunum expressing the highest levels of mRNA and much lower levels of message observed in colon, cecum, and rectum. A prominent GPR66/FM-3 band was observed at 2.4 kilobases. Some larger messages were also detected (particularly in jejunum) that most likely result from differing polyadenylation. The overlapping expression of both NmU and GPR66/FM-3 in the upper GI tract implies that this receptor-ligand pair plays a role in regulating the function of this organ system.

Given that GPR66/FM-3 was originally cloned from T lymphocytes and also that we had observed significant expression of GPR66/FM-3 in peripheral blood leukocytes and spleen, the expression of both NmU and its receptor in the immune system was examined in greater detail using quantitative PCR (Fig. 4). Interestingly, among the various immune cell types examined (including monocytes, dendritic cells, mast cells, epithelial cells, and various lymphocytes), significant levels GPR66/FM-3 expression was detected in only NK cells and T cells (Fig. 4). In contrast, NmU was expressed in dendritic cells, monocytes, and B cells. The pattern of NmU and GPR66/FM-3 expression observed in lymphoid cells is intriguing and suggests a possible interaction between helper/antigen-presenting cell types that express NmU and effector populations that express the receptor. The high expression of NmU and its receptor in the small intestine, and in particular the expression of the receptor in T cells, suggests the potential involvement of NmU in mucosal immunity. Further studies will be necessary to determine what, if any, role neuromedin U plays in regulating the immune response.

In summary, we have shown that the previously orphan GPCR GPR66/FM-3 is a specific receptor for NmU. Cells transfected with GPR66/FM-3 show a dose-dependent response to NmU with an EC_{50} of 10 nM and do not respond to other neuromedins including neuromedin C (Fig. 2), B and N (data not shown), as well as the kassinin-like tachykinins neurokinin A and B (data not shown). We have further shown that although NmU is broadly expressed, GPR66/FM-3 expression is relatively limited and that the ligand and receptor overlap only in the upper GI tract. In addition, we have expanded the analysis of NmU and GPR66/FM-3 into cells and tissues of the immune system. The data from these expression studies suggest a role for NmU in immune regulation that was not previously appreciated. Further genetic and biochemical analysis will be necessary to fully define the downstream signaling pathways and physiological role(s) of this newly discovered ligand-receptor system.

References

Augood SJ, Keast JR and Emson PC (1988) Distribution and characterisation of neuromedin U-like immunoreactivity in rat brain and intestine and in guinea pig intestine. *Regul Pept* **20:**281–292.

Austin C, Lo G, Nandha KA, Meleagros L and Bloom SR (1995) Cloning and characterization of the cDNA encoding the human neuromedin U (NmU) precursor: NmU expression in the human gastrointestinal tract. *J Mol Endocrinol* **14:**157–169.

Ballesta J, Carlei F, Bishop AE, Steel JH, Gibson SJ, Fahey M, Hennessey R, Domin J, Bloom SR and Polak JM (1988) Occurrence and developmental pattern of neuromedin U-immunoreactive nerves in the gastrointestinal tract and brain of the rat. *Neuroscience* **25:**797–816.

Benito-Orfila MA, Domin J, Nandha KA and Bloom SR (1991) The motor effect of neuromedin U on rat stomach in vitro. *Eur J Pharmacol* **193:**329–333.

Bockman CS, Abel PW, Hicks JW and Conlon JM (1989) Evidence that neuromedin U may regulate gut motility in reptiles but not in mammals. *Eur J Pharmacol* **171:**255–257.

Brown DR and Quito FL (1988) Neuromedin U octapeptide alters ion transport in porcine jejunum. *Eur J Pharmacol* **155:**159–162.

Chambers J, Ames RS, Bergsma D, Muir A, Fitzgerald LR, Hervieu G, Dytko GM, Foley JJ, Martin J, Liu WS, Park J, Ellis C, Ganguly S, Konchar S, Cluderay J, Leslie R, Wilson S and Sarau HM (1999) Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature (Lond)* **400:**261–265.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275:**10767–10771.

Conlon JM, Domin J, Thim L, DiMarzo V, Morris HR and Bloom SR (1988) Primary structure of neuromedin U from the rat. *J Neurochem* **51:**988–991.

Coward P, Chan SD, Wada HG, Humphries GM and Conklin BR (1999) Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. *Anal Biochem* **270:**242–248.

Domin J, Benito-Orfila MA, Nandha KA, Aitken A and Bloom SR (1992) The purification and sequence analysis of an avian neuromedin U. *Regul Pept* **41:**1–8.

Domin J, Ghatei MA, Chohan P and Bloom SR (1987) Neuromedin U—a study of its distribution in the rat. *Peptides* **8:**779–784.

Feighner SD, Tan CP, McKee KK, Palyha OC, Hreniuk DL, Pong SS, Austin CP, Figueroa D, MacNeil D, Cascieri MA, Nargund R, Bakshi R, Abramovitz M, Stocco R, Kargman S, O'Neill G, Van Der Ploeg LH, Evans J, Patchett AA, Smith RG and Howard AD (1999) Receptor for motilin identified in the human gastrointestinal system. *Science (Wash DC)* **284:**2184–2188.

Gardiner SM, Compton AM, Bennett T, Domin J and Bloom SR (1990) Regional hemodynamic effects of neuromedin U in conscious rats. *Am J Physiol* **258:**R32–R38.

Honzawa M, Sudoh T, Minamino N, Tohyama M and Matsuo H (1987) Topographic localization of neuromedin U-like structures in the rat brain: An immunohistochemical study. *Neuroscience* **23:**1103–1122.

Kage R, O'Harte F, Thim L and Conlon JM (1991) Rabbit neuromedin U-25: Lack of conservation of a posttranslational processing site. *Regul Pept* **33:**191–198.

Lo G, Legon S, Austin C, Wallis S, Wang Z and Bloom SR (1992) Characterization of complementary DNA encoding the rat neuromedin U precursor. *Mol Endocrinol* **6:**1538–1544.

Maggi CA, Patacchini R, Giuliani S, Turini D, Barbanti G, Rovero P and Meli A (1990) Motor response of the human isolated small intestine and urinary bladder to porcine neuromedin U-8. *Br J Pharmacol* **99:**186–188.

Malendowicz LK, Nussdorfer GG, Markowska A, Tortorella C, Nowak M and Warchol JB (1994) Effects of neuromedin U (NMU)-8 on the rat hypothalamo-pituitary-adrenal axis. Evidence of a direct effect of NMU-8 on the adrenal gland. *Neuropeptides* **26:**47–53.

Malendowicz LK, Nussdorfer GG, Nowak KW and Mazzocchi G (1993) Effects of neuromedin U-8 on the rat pituitary-adrenocortical axis. *In Vivo* **7:**419–422.
Minamino N, Kangawa K, Honzawa M and Matsuo H (1988) Isolation and structural determination of rat neuromedin U. *Biochem Biophys Res Commun* **156:**355–360.
Minamino N, Kangawa K and Matsuo H (1985a) Neuromedin U-8 and U-25: Novel uterus stimulating and hypertensive peptides identified in porcine spinal cord. *Biochem Biophys Res Commun* **130:**1078–1085.
Minamino N, Sudoh T, Kangawa K and Matsuo H (1985b) Neuromedins: Novel smooth-muscle stimulating peptides identified in porcine spinal cord. *Peptides* **6 (Suppl 3):**245–248.
Murphy R, Turner CA, Furness JB, Parker L and Giraud A (1990) Isolation and microsequence analysis of a novel form of neuromedin U from guinea pig small intestine. *Peptides* **11:**613–617.
O'Harte F, Bockman CS, Abel PW and Conlon JM (1991) Isolation, structural characterization and pharmacological activity of dog neuromedin U. *Peptides* **12:**11–15.
Salmon AL, Johnsen AH, Bienert M, McMurray G, Nandha KA, Bloom SR and Shaw C (2000) Isolation, structural characterization and bioactivity of a novel neuromedin U analog from the defensive skin secretion of the Australasian tree frog, Litoria caerulea. *J Biol Chem* **275:**4549–4554.
Sullivan E, Tucker EM and Dale IL (1999) Measurement of [Ca^{2+}] using the Fluorometric Imaging Plate Reader (FLIPR). *Methods Mol Biol* **114:**125–133.
Sumi S, Inoue K, Kogire M, Kogire D, Takaori K, Suzuki T, Yajima H and Tobe T (1987) Effect of synthetic neuromedin U-8 and U-25, novel peptides identified in porcine spinal cord, on splanchnic circulation in dogs. *Life Sci* **41:**1585–1590.
Tan CP, McKee KK, Liu Q, Palyha OC, Feighner SD, Hreniuk DL, Smith RG and Howard AD (1998) Cloning and characterization of a human and murine T-cell orphan G-protein-coupled receptor similar to the growth hormone secretagogue and neurotensin receptors. *Genomics* **52:**223–229.

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