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Novel orphanin FQ/nociceptin transcripts are expressed in human immune cells

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Abstract

The opioid-like receptor (NOP) is widely expressed throughout the human immune system. Here, we report that human peripheral blood lymphocytes (PBLs) express transcripts encoding the NOP receptor agonist, orphanin FQ/nociceptin (OFQ/N). OFQ/N transcripts in resting PBLs were restricted to CD19 + B cells and contained a novel 5′ exon (ImEx2b), replacing exons 1 and 2 found in neuronal transcripts. Translation of ImEx2b-containing transcripts resulted in truncated OFQ/N precursors lacking a classical signal peptide. Mitogen activation of PBLs dramatically up-regulated neuronal-like transcripts, predominantly in CD3 + T cells. Overall, this suggests different promoters direct specific OFQ/N transcript expression in immune cells.

Keywords: ORL-1; NOP; Orphanin FQ; Nociceptin; B lymphocytes; Alternative splicing

1. Introduction

Orphanin FQ or Nociceptin (OFQ/N) is a neuropeptide that has been implicated in the mediation of pain sensation and auditory perception, as well as complex behaviors such as lordosis, locomotion, learning and anxiety (reviewed by Harrison and Grandy, 2000). The 17-amino acid peptide is derived from a larger precursor encoded by a transcript derived from four exons (Mollereau et al., 1996). OFQ/N mediates its actions via a G protein coupled receptor termed the NOP receptor, also known as ORL-1 (Mollereau et al., 1994). The NOP receptor has striking similarity to the classical opioid receptors (mu, delta and kappa) both in primary sequence, as well as in secondary messenger activation. NOP receptor activation of Go/i subunits leads to the inhibition of adenylate cyclase, activation of MAPK, inhibition of Ca^{2+} conductance and potentiation of K^{+} currents (Connor et al., 1996a,b; Hawes et al., 1998; Matthes et al., 1996; Meunier et al., 1995; Reinscheid et al., 1995).

The NOP receptor expression in immune cells has been well documented. Halford et al. (1995) demonstrated that mouse peripheral blood lymphocytes (PBLs) express NOP receptor transcripts following mitogen activation. Other studies demonstrated NOP expression in human immune tissues, PBLs and numerous cell lines (Miotto and Evans, 1996; Wick et al., 1995) and that the RNA levels for the NOP receptor in human mixed lymphocytes or selected cell lines were comparable to those of the human central nervous system (Peluso et al., 1998). NOP expression in immune cells was also demonstrated with ligand binding studies (Hom et al., 1999; Serhan et al., 2001), as well as agonist-stimulated GTPγS binding experiments (Peluso et al., 2001). Furthermore, NOP receptor expression has been implicated in the regulation of immune function such as antibody production (Halford et al., 1995), modulation of cAMP in human B lymphocyte cell line (Raji) (Hom et al., 1999) and neutrophil chemotaxis (Serhan et al., 2001).

Due to the previous findings that established the expression of NOP receptor in immune cells, it was of interest to determine if human immune cells were also capable of synthesizing the NOP receptor ligand OFQ/N. We have previously demonstrated that human neuronal OFQ/N transcripts can be alternatively spliced to generate a total of four possible messages (Arjomand and Evans, 2001). Of these,

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the two longer transcripts were demonstrated to be conserved in both mouse and rat, whereas the shorter transcripts were novel in human and lacked exon 2 which contains the putative translational start site and signal peptide. Based on these findings, we sought to determine whether human PBLs expressed OFQ/N transcripts, and if so, identify the various splice variants. Here we report that upon activation, human PBLs express OFQ/N messages, while resting PBLs express novel OFQ-like messages that utilize a novel 5′exon, and presumably, an alternative transcriptional start site and promoter. Further analyses of sorted PBLs revealed that expression of these novel transcripts were limited to CD19 + B lymphocytes.

2. Materials and methods

2.1. Cell culture

Human peripheral blood lymphocytes (PBLs) were isolated by Ficoll gradient centrifugation and cultured in RPMI containing 10% human serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 200 U/ml of recombinant human IL-2 at 37 °C and 5% CO_2. PBL activation was induced with 1 μg/ml of phytohemagglutinin (PHA). Leukocyte subsets were isolated by immunomagnetic positive selection for CD3+, CD4+, CD8+, CD14+, CD19+, CD56+ cells (Miltenyi Biotec, Auburn, CA).

CEM, THP, Jurkat and Ramos cell lines were cultured in RPMI containing 10% fetal bovine serum (Omega,Tarzana, CA), 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 25 ng/ml fungizone and 10 mM HEPES (pH 7.4) at 37 °C with 5% CO_2. Epstein–Barr virus transformed B cells were generously provided by Dr. Janis Giorgi.

Human tissues were obtained from the UCLA human tissue bank in accordance to the UCLA Office of Protection of Research Subjects (HSPC #G97-10-013-02). Freshly isolated PBLs were obtained from the UCLA core facility. Following treatment or sorting of PBLs, all tissues were stored at − 70 °C and total RNA was extracted using either Trizol (Life Technologies, Rockville, MD) or RNeasy mini kits (Qiagen, Valencia, CA). When feasible, RNA integrity was assessed by gel electrophoresis. RNA concentrations were measured spectrophotometrically.

2.2. Ribonuclease protection assay (RPA)

Nuclease protection assays were performed as previously described (Arjomand and Evans, 2001). Briefly, a 456-bp fragment, corresponding to a segment spanning exons 2 and 3 of the human OFQ/N message, was used as a template for cRNA probe synthesis. Protection assays were conducted with HybSpeed RPA kit (Ambion, Austin, TX) according to manufacturer's suggested protocols along with total RNA extracted from either human biopsy tissues or PBLs. The samples were electrophoresed in 5% acrylamide gels and exposed overnight to X-OMAT AR film (Eastman Kodak, Rochester, NY) at − 70 °C with an intensifying screen.

2.3. RT-PCR

One or two micrograms of total RNA was reverse transcribed using 125 ng of random hexamers and 200 U of M-MLV reverse transcriptase (Promega, Madison, WI) or Superscript 2 (Stratagene, La Jolla, CA). Polymerase chain reaction (PCR) amplification was carried out using 1/10 of the cDNA, 2.5 mM $MgCl_2$, 200 μM dNTP, 20–25 pmol of primer pairs listed in Table 1, and 1.25–2.5 U of Hot Start *Taq* or *Taq* polymerase (Qiagen).

Amplification was carried out in a GeneAmp 2400 cycler (Perkin-Elmer, Foster City, CA) using a Touchdown-Hot Start PCR protocol, as previously described (Arjomand and Evans, 2001). Briefly, following an initial 15-min denaturation at 94 °C, cycling was carried out at 94 °C for 30 s, extensions at 72 °C for 1 min and a final 7-min extension at 72 °C. These parameters were kept constant while the annealing temperature was reduced from 60 to 50 °C, with three cycles each at 60, 57, 54 and 52 °C and 25 cycles at 50 °C. PCR products were separated by electrophoresis on 1.5 % agarose gels in 1 × TAE or 1 × TBE, stained with ethidium bromide and visualized by UV illumination.

2.4. Rapid amplification of cDNA ends (RACE)

Human spleen mRNA was isolated using PolyATtract (Promega) and 3 μg poly(A+) RNA was used with the Marathon cDNA amplification Kit (Clontech, Palo Alto, CA) as suggested by the manufacturers. Five micrograms of total RNA from a Burkitts lymphoma cell line (Ramos, ATCC number CRL-1596) was used for RNA ligase-mediated (RLM) RACE amplification with GeneRacer

Table 1
PCR primers

Exon	Sense	Sequence (5′–3′)
ImEx2b	+	GGC TCC AAA AAC CCG ACT AAA G
Ex1	+	GAT ATA TAT GCT GGT GTG GCT G
Ex2	+	*GGA TCC* GCA CCA TGA AAG TCC TGC TTT G
Ex4	–	AGC AAC AGG GTT GTG GTG AAA C
RaceEx1	–	CCC TCC GAT GGA TGC GAG
RaceEx3	–	AGA GGG GGC TGG GGA AGA C
ORL-1	+	CCG CAT CTG CTG CTC AAT GC
ORL-1	–	GAT CAT GAG GCT GTA GCA GA
β-Actin	+	TAC AAC CTC CTT GCA GCT CC
β-Actin	–	TCT TCA TGA GGT AGT CTG TC

Italicized bases correspond to a *Bam*H1 restriction site.

Kit (Invitrogen, Carlsbad, CA). Internal OFQ/N primers used for the amplification are listed in Table 1.

2.5. Northern blot

A human immune tissue blot (Clontech) was probed with a [^{32}P]-end labeled oligonucleotide probe complementary to the novel immune exon (ImEx2b in Table 1) as previously described (Sambrook et al., 1989). The blot was exposed to a phosphoimager screen (Molecular Dynamics, Sunnyvale, CA).

2.6. Library screening

The Ramos human B cell cDNA library was generously provided by Dr. Jim Boulter and screened as described elsewhere (Sambrook et al., 1989). The probe used for screening the Ramos library was an oligomer (5′TTTGGGGGCTTCACCGGGC3′), corresponding to portion of the OFQ/N exon 3 that encodes for the OFQ/N peptide. The probe was end labeled with P^{32}γATP (ICN, Costa Mesa, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), hybridized overnight at 42 °C in a buffer containing 1 M NaCl, 50 mM Tris (pH 7.5), 5× Denhardt's, 0.1% sodium pyrophosphate, 0.5% SDS and 10% (w/v) PEG-8000. High stringency washes were carried out with several rinses with 0.1×SSPE, 0.1% SDS at 37 °C and the filters exposed overnight to Kodak X-OMAT AR film at −70 °C with an intensifying screen.

2.7. Subcloning and sequencing

PCR fragments were extracted from agarose gels using Qiagen gel extraction kit (Qiagen) and, in most cases, ligated into PCRscript (Stratagene) vector for sequencing or cRNA synthesis. Sequencing was performed with either Sequenase version 2.0 (Amersham, Piscataway, NJ) or at the UCLA sequencing facility. Sequence analyses were performed with MacVector (Scientific Imaging Systems, New Haven, CT) and/or web-based search engines (NCBI, TESS: URL: http://www.cbil.upenn.edu/tess (Schug and Overton, 1997)).

2.8. In vitro translation

Vectors containing fragments of interest were linearized with restriction enzymes (New England Biolabs) and sense RNA was synthesized using either T3 or T7 polymerase (Promega) depending on orientation. Following phenol–chloroform extraction and ethanol precipitation, the RNA was translated in vitro with [^{35}S]-methionine using Retic Lysate IVT (Ambion). The resulting reactions were electrophoresed 15% polyacrylamide gels, fixed with 10% acetic acid and 45% methanol, dried and exposed using a phosphoimager screen (Molecular Dynamics).

3. Results

3.1. RPA analyses

3.1.1. Human spleen expresses OFQ/N transcripts lacking exon 2

We have previously described the existence of low abundant, alternatively spliced OFQ/N messages in human brain whereby exon 2, which encodes the translational start site, was deleted (Arjomand and Evans, 2001). The RPA probe was designed to distinguish the two alternatively spliced forms of OFQ/N transcripts by spanning portions of exons 2 and 3 (Fig. 1A). A protected fragment of 457 nt corresponds to transcripts containing exon 2, whereas a 326-

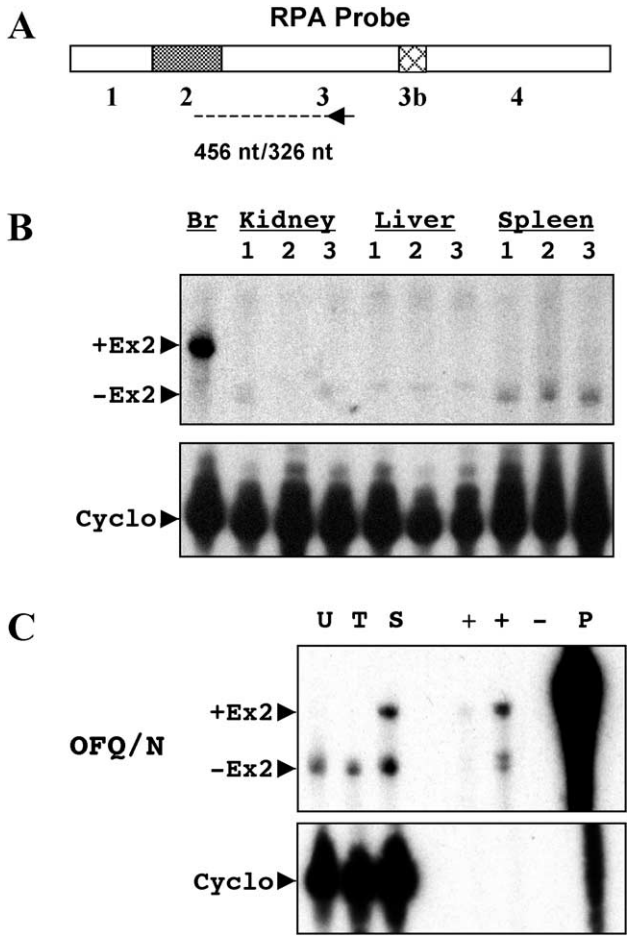

Fig. 1. Immune tissues and cells express OFQ/N transcripts. (A) Schematic of the cRNA probe used in the RPA experiments. Full protection of the probe would result in a 456-nt band while spliced messages without exon 2 (shaded region) would be truncated to 326 nt. (B) RPA analysis of various human tissues. Human cortex (15 μg) or RNA (30 μg) from various organs from independent donors were used with the OFQ/N probe (top panel) or cyclophilin control (bottom panel); Cortex (Br). (C) RPA analysis of PBLs ± PHA. RNA (30 μg) from unstimulated (U), 48-h control (T) or 48-h 1 μg/ml PHA-treated PBLs was used with the OFQ/N probe (top panel) or Cyclophilin probe (bottom panel). (+)/(+) 100/500 fg sense cRNA from exon 2 containing and exon 2 minus OFQ/N plasmids; (−) 50 μg yeast RNA digestion control; undigested probe (P).

nt protected fragment corresponds to OFQ/N transcripts missing exon 2. Fig. 1B demonstrates that human spleen expresses OFQ/N transcripts lacking exon 2, while the positive control, human cortex, expresses full-length OFQ/N messages. OFQ/N transcripts were not detected in either kidney or liver.

3.1.2. Activated PBLs express exon 2 containing OFQ/N transcripts

Using RPA assays, OFQ/N transcripts lacking exon 2 were detected in both resting and PHA-activated PBLs. However, following 48 h of PHA stimulation, the activated PBLs are shown to also express exon 2 containing OFQ/N transcripts (Fig. 1C). The expression of OFQ/N transcripts was further confirmed with RT-PCR using primers spanning exons 2–4. In the two independent samples shown, the amplified fragments from the PHA treated PBLs, correspond to OFQ/N transcripts with and without exon 3b (Fig. 2A, top panel), as previously described for mouse, rat and human CNS (Arjomand and Evans, 2001; Saito et al., 1996). To determine which subset(s) of activated PBLs expressed neuronal OFQ/N transcripts, we used the aforementioned RT-PCR protocol, subsequent to cell sorting. In Fig. 2B (top panel), the strongest OFQ/N signal is observed from the monocyte depleted fraction (14-F/T) and CD3 + T lymphocyte population. Minor expression was also observed in downstream fractions, as well as 19 + 56+ (B lymphocyte and NK cell pooled cells). β-Actin was used to control for the RT reaction (Fig. 2A and B, lower panels).

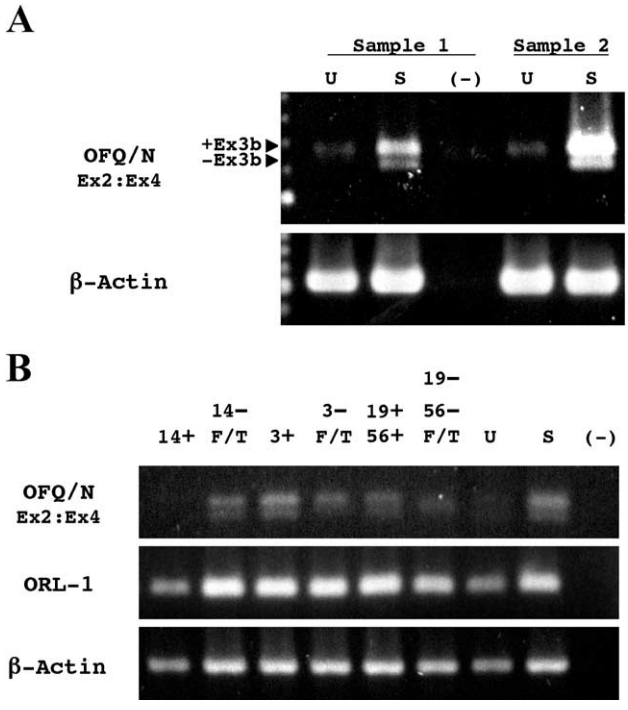

Fig. 2. RT-PCR of PBLs ± PHA. (A) Two micrograms of total RNA from independent PBLs cultured for 48 h with (S) or without (U) 1 μg/ml PHA were used for RT-PCR using primers spanning exons 2–4 of OFQ/N (top panel) or β-actin (bottom panel). (B) One microgram of total RNA from sorted or depleted PHA-activated PBLs pools were used for RT-PCR. The top panel uses primer pair Ex2 and Ex4 of the OFQ/N transcript. The middle panel is specific for the NOP receptor using the ORL-1 primer pair. The bottom panel serves as a control for the RT reaction using the β-actin primer pair. CD14 + monocytes (14+), CD14 + depleted flow through (14-F/T), CD3 + T lymphocytes (3+), CD3 + depleted flow through (3-F/T), CD19 + B lymphocytes and CD56 + NK cells (19 + 56+), CD19+ and CD56+ depleted flow through (19 − 56 −), unstimulated PBLs (U), PHA stimulated PBLs (S), PHA-stimulated PBLs from which the RT reaction was omitted (−).

3.2. 5′ RACE and RT-PCR of tissues and cell lines

3.2.1. Immune tissues express a novel OFQ/N exon

In light of the RPA results regarding immune expression of OFQ/N transcripts lacking exon 2, we performed additional RT-PCR experiments using primers from exons 1 and 4. Previously, we had shown minor expression of alternatively spliced OFQ/N transcript devoid of exon 2 in human cortex (Arjomand and Evans, 2001). If the shorter immune OFQ/N transcripts were analogous to the neuronal transcripts lacking exon 2, then such RT-PCR should have resulted in the amplification of two bands of 716 and 658 bp in length, depending on the splicing patterns between exons 3 and 4. We were unable to amplify such fragments from spleen or freshly isolated PBLs (data not shown), suggesting that these transcripts could be missing exons 1 or 4.

In order to characterize the upstream sequences of the OFQ/N transcripts lacking exon 2, we performed 5′ RACE experiments using mRNA isolated from human spleen and downstream primers located in exons 1 or 3. Consistent with a transcript missing exon 1, only the RACE experiments using exon 3 primers resulted in the amplification of approximately 150-bp bands (data not shown). The resulting fragments were subcloned, sequenced and compared to GenBank sequences. Positive matches were obtained with the EST database. Specifically, a clone derived from human germinal B cells (accession number AA282862) matched 108 bp of the RACE sequence, as well as OFQ/N exon 3 sequence (Fig. 3B). Analyses of the genomic region upstream to exon 3 (accession number AA885285) revealed that the 108 bp corresponds to a stretch of intronic sequence 251 bp 5′ of the start of exon 3. The transcriptional start site(s) of the immune-derived OFQ/N messages was determined using RLM-RACE and yielded a novel exon 176 bp in length, which we call ImEx2b. A schematic of the updated OFQ/N gene structure and immune splice variants is depicted in Fig. 3A.

To confirm the expression and use of this potential exon, a commercial immune tissue Northern blot was probed using a PCR generated [^{32}P]-labeled probe corresponding to ImEx2b (Fig. 4). Exposure of the blot revealed the expression of an approximately 1100-bp message in spleen, lymph nodes, appendix and PBL. Minor expression was also noted in bone marrow. A less intense signal of 5.1 kb was also detected in the aforementioned tissues. No expression was detected in the thymus, an area important for T cell development.

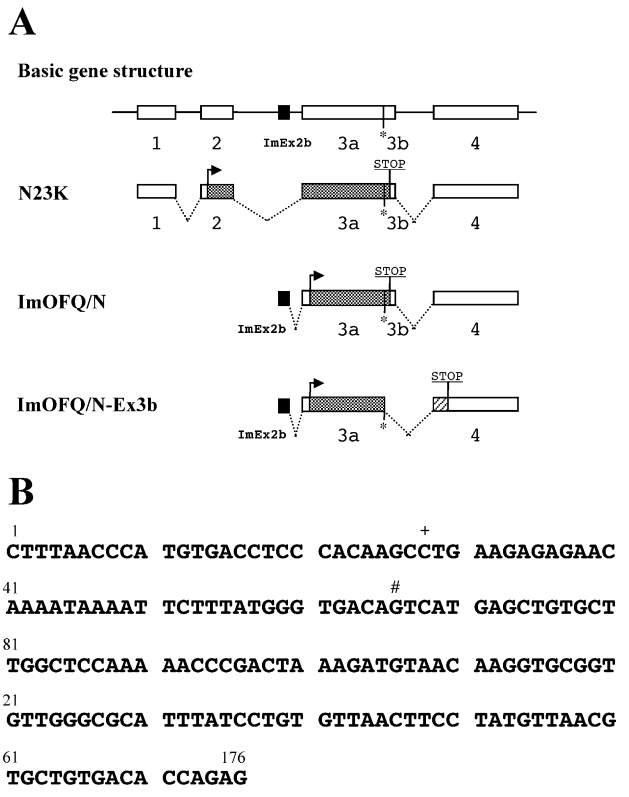

Fig. 3. Updated gene structure and splicing pattern of the immune-derived OFQ/N. (A) The human OFQ/N exons are numbered sequentially with the novel immune exon depicted by a black box (ImEx2b). N23K represents the classical human neuronal OFQ transcript. The arrow represents the translational start site and the hatched region the open reading frame. The immune transcripts begin with ImEx2b and span exons 3 and 4. The asterisk represents the start of exon 3b that is alternatively spliced in the shorter transcripts, thereby extending the open reading frame into exon 4. (B) Sequence of the 176-bp immune-derived OFQ/N exon (ImEx2b) obtained with RLM-RACE from Ramos cell line. Alternative transcriptional start site (+), transcript equivalent to GenBank accession number AA282862 (#).

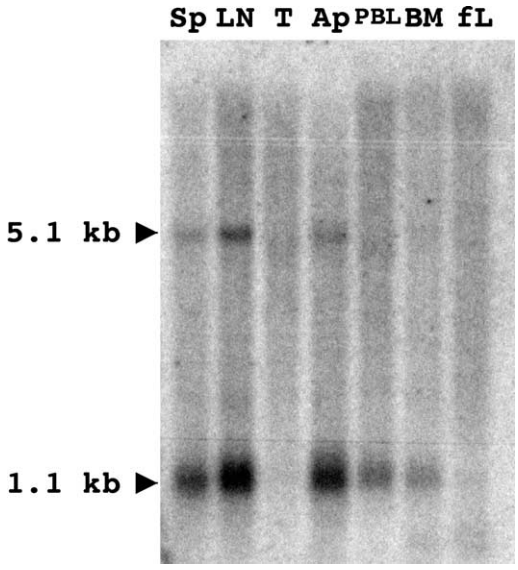

Fig. 4. Northern blot of the various human tissues. A PCR-based probe complementary to ImEx2b was hybridized to a commercial Northern blot. Spleen (Sp), lymph nodes (LN), thymus (T), appendix (Ap), peripheral blood lymphocytes (PBL), bone marrow (BM), fetal liver (fL).

RT-PCR experiments using upstream primers complementary to ImEx2b and OFQ/N exon 4 (Fig. 5A, solid line) resulted in the amplification of two bands from spleen (Fig. 5B). The sequence of the longer fragment corresponded to ImEx2b and exons 3–4, whereas the shorter fragment corresponded to the same sequence lacking exon 3b. Similar RT-PCR with human brain or liver, resulted in negligible amplification of the novel transcripts. Human brain cDNA was also amplified using primers spanning exons 2–4 of OFQ/N (Fig. 5A, dashed line), which resulted in the expected amplification of 767- and 709-bp fragments (Lane 1, Fig. 5B).

3.2.2. B cells selectively express the immune OFQ/N transcripts

In order to further characterize the distribution of the novel immune-derived OFQ/N transcripts, we performed RT-PCR experiments using a variety of human immune cell lines. These included a macrophage cell line (THP), two T cell lines (CEM and Jurkat) and two B cell lines (Ramos and EBV transformed B cells). Only the B cell lines resulted in the amplification of the novel transcripts analogous to the spleen derived messages (data not shown). To determine which subset of primary immune cells express the immune

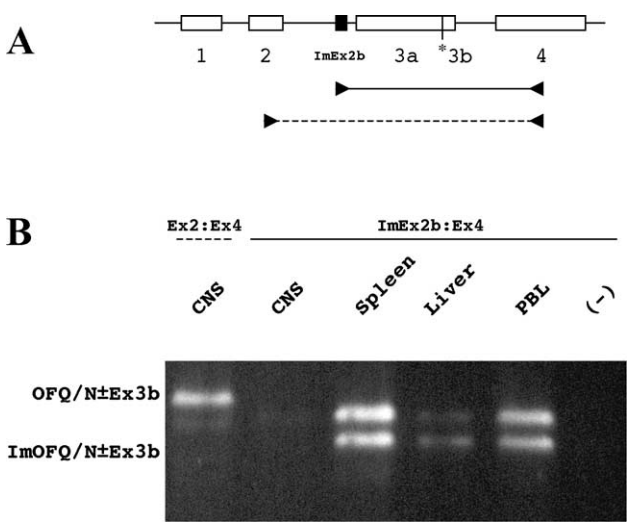

Fig. 5. Expression of immune-derived OFQ/N in various human tissues. (A) The solid line represents the location of the primer pairs spanning the immune specific transcripts. The dashed line represents the location of the primer pairs spanning the classic neuronal OFQ/N transcripts. (B) RT-PCR was performed using 2 μg total RNA from the tissues listed using the primer pairs depicted in (A). Human brain (CNS), peripheral blood lymphocytes (PBL), PBL from which the RT reaction was omitted (–).

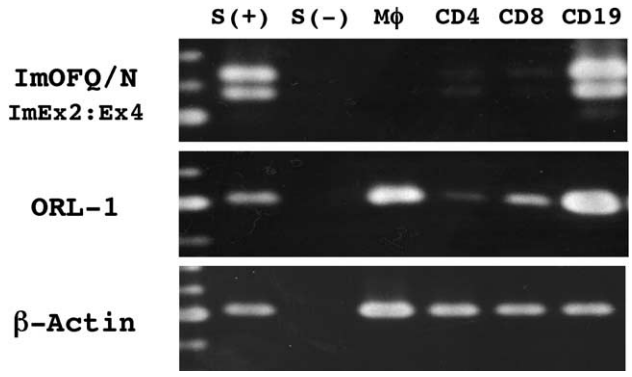

Fig. 6. Immune-derived OFQ/N is expressed in B cells. RT – PCR was performed using various primer pairs listed in Table 1 and 1–2 μg total RNA from spleen or sorted PBMC. A total of three to four pools of PBMC from independent donors were sorted. The figure shown is a representative of one of these experiments. The top panel uses primer pairs ImEx2b to Ex4 and is specific for immune-derived OFQ/N transcripts. The middle panel is specific for the NOP receptor using the ORL-1 primer pair. The bottom panel serves as a control for the RT reaction. Spleen (S(+)), spleen from which the RT reaction was omitted (S(–)), macrophage (Mϕ), CD4+T lymphocytes (CD4), CD8+cytotoxic T lymphocytes (CD8), CD19+B lymphocytes (CD19).

OFQ/N, RT-PCR was performed on PBL subsets isolated by immunomagnetic selection. The top panel of Fig. 6 is a representative experiment demonstrating the expression of immune OFQ/N transcripts only in CD19+B cells. Neither CD14+ (monocyte marker), CD4+ or CD8+ (T cells), nor CD56+ (NK cells) express the immune OFQ/N (NK cell data not shown).

3.2.3. NOP receptor expression in sorted PBLs

We also analyzed the sorted PBLs for the expression of the NOP receptor using RT-PCR. In freshly isolated PBLs, NOP expression was variable across donors especially for the T cell subsets. However, NOP was consistently detectable (Fig. 6, middle panel). In PHA-activated PBLs, NOP expression was easily detectable in all sorted cell types (Fig. 2B, middle panel). β-Actin was used as an internal control for the verification of proper reverse transcription and RNA integrity (Figs. 2B and 6, bottom panels).

3.3. Ramos cDNA library screen and in vitro translation (IVT)

3.3.1. Immune OFQ/N transcripts are translated from an exon 3 translational start site

We sought to obtain full-length clones of the immune OFQ/N by screening a human B cell Ramos library using an oligomer from exon 3. Two clones representing the immune OFQ/N ± Exon3b were identified and sequenced. These sequences matched those of the human spleen 5′ RACE clones but included only 109 bp of ImEx2b. However, since clones derived from cDNA libraries can have incomplete 5′ ends, we also analyzed Ramos total RNA using RNA-ligase mediated 5′ RACE (RLM-RACE) which is designed to only amplify full length or capped transcripts. Sequence analysis of RLM-RACE products resulted in the characterization of longer sequences from two potential transcriptional start sites. The longest transcripts included a novel exon 176 bp in length (Fig. 3B).

Analysis of these sequences for potential open reading frames (ORF) resulted in a putative translational start site with a favorable Kozac consensus sequence, located in exon 3 (Kozak, 1996). This ORF is in frame with the preproOFQ/N precursor but lacks a characteristic hydrophobic amino terminal signal sequence indicative of secretory proteins. These ORFs are identical to previously described neuronal OFQ/N alternative splice variants devoid of exon 2 previously isolated from human CNS (Arjomand and Evans, 2001).

The two Ramos clones isolated from the cDNA library screen were linearized with appropriate restriction enzymes for in vitro translation assays. Previously obtained human brain OFQ/N splice variants devoid of exon 2 were included for comparison. Translation of the immune clones resulted in proteins having a molecular mass of approximately 16.5 and 20 kDa. The size differences result from the splicing of the exon 3b that contains the putative stop codon (the longer products represent spliced transcripts with extended translation into exon 4). Analogous migration patterns were obtained from the in vitro translation of neuronal OFQ/N transcripts lacking exon 2 (Arjomand and Evans, 2001) (Fig. 7).

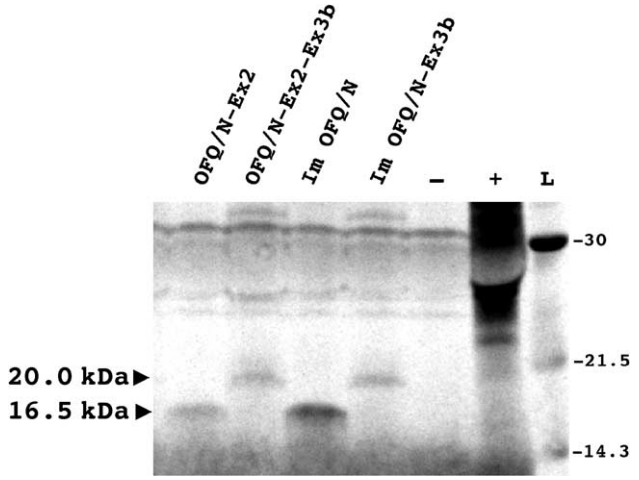

Fig. 7. In vitro translation of the immune-derived OFQ/N transcripts. Alternatively spliced, neuronally derived OFQ/N clones lacking exon 2 or clones isolated from the Ramos library were linearized, in vitro transcribed using either T3 or T7 polymerase, and the resulting cRNA translated with reticulocyte lysate in the presence of [^{35}S]-methionine. (–) IVT reaction without RNA, (+) positive control *Xenopus* elongation factor-1, (L) [^{14}C]-labeled molecular size marker.

4. Discussion

The opioid system has been implicated to play a role in regulation and maintenance of the immune system by modulating factors such as antibody and cytokine production, phagocytosis, chemotaxis and cellular proliferation and differentiation (reviewed by Salzet et al., 2000; McCarthy et al., 2001). In addition, immune-derived opioid peptides have been shown to induce analgesia at the site of inflammation (Mousa et al., 2001; Stein, 1995). Hence, the opioid system may not only function as an immune modulator but immune-derived opioid peptides may also be involved in mediating neuronal functions such as pain perception.

The NOP receptor, which shares considerable homology in sequence and function to the classical opioid receptors, has been shown to be expressed in immune cells (Halford et al., 1995; Hom et al., 1999; Miotto and Evans, 1996; Peluso et al., 1998; Serhan et al., 2001; Wick et al., 1995). Interestingly, buprenorphine, used clinically as an analgesic and as a maintenance therapy for opioid abusers, has been shown to be a partial agonist of the NOP receptor (Bloms-Funke et al., 2000). Thus, it is conceivable that buprenorphine may directly modulate immune cells via the NOP receptor.

The presence of NOP receptor expression in immune cells prompted us to determine if the immune system was also capable of synthesizing the NOP receptor ligand, OFQ/N. Here, we report that mitogen activation of human PBL results in the detection of OFQ/N transcripts analogous to the murine neuronal transcripts N23K and N27K and that their expression is predominantly in CD3+ (T lymphocytes) (Saito et al., 1996). Upon translation, these transcripts would yield preproOFQ/N precursors, encoding multiple bioactive peptides (Florin et al., 1997; Lee et al., 1999; Okuda-Ashitaka et al., 1998). Such immune-derived precursor proteins would possess signal peptide sequences and presumably be targeted to the secretory pathway in immune cells. The processing and release of the bioactive peptide(s) would depend on the cohort of proteolytic enzymes present in the OFQ/N expressing immune cells (reviewed by Terenius et al., 2000).

The upregulation of OFQ/N transcripts coincide with the reported appearance of NOP receptor transcripts following activation (Wick et al., 1995). It may be postulated that OFQ/N acts as a paracrine or autocrine factor for immune cells. Alternatively, immune-derived OFQ/N may act as a neuroimmune signal exerting its effects on peripheral nerves expressing NOP receptors (Jia et al., 1998; reviewed by Mollereau and Mouledous, 2000).

Sorted PBL subsets were also used to determine which cell types may express the NOP receptor. In nonstimulated cells, NOP expression levels were inconsistent but nonetheless detectable in all cell types tested. The varying results obtained for NOP expression in sorted PBL from our donors could be a result of their differential states of immune activation. This would be consistent with the pronounced expression of NOP transcripts detected in our activated and sorted PBL samples, as well as others reporting that activated immune cells upregulate NOP message levels (Halford et al., 1995; Wick et al., 1995).

RPA analyses from human immune tissues and PBLs revealed expression of OFQ/N transcripts lacking exon 2 in both resting and activated PBLs. Although we had previously described alternative splicing of human neuronal OFQ/N messages lacking exon 2 (Arjomand and Evans, 2001), the immune-derived transcripts failed to amplify with primers spanning exons 1–4. This prompted us to perform 5′ RACE of human spleen in order to determine the nature of the 5′ termini of the truncated OFQ/N messages, which resulted in the isolation of a novel exon located 254 bp upstream to the exon 3 start site. Northern blot analysis of immune tissues using a probe specific for this novel exon revealed a 1.1-kb message, as opposed to a 1.3-kb message observed in neuronal tissues (Mollereau et al., 1996; Nothacker et al., 1996). In the PBL subsets tested, the novel immune transcripts appear to be limited to CD19+B lymphocytes. A cDNA library screen of the human B cell lymphoma line (Ramos) resulted in the isolation of two clones differing in their splicing patterns of exon 3b. We and others have shown that the splicing of the 3′ region of exon 3 (exon 3b) results in the elimination of a stop codon and extends the translation of the carboxy terminal of preproOFQ/N into exon 4 by 25 amino acids (Arjomand and Evans, 2001; Saito et al., 1996). In vitro translation of the Ramos derived clones resulted in the synthesis of analogous protein precursors to those of neuronal OFQ/N splice variants missing exon 2 (Arjomand and Evans, 2001). Since the Ramos transcripts only have exons 3 and 4 in common with the neuronal counterparts, translation must have been initiated from an alternative translational start site located within exon 3. These precursor proteins would thus lack the signal peptide sequence found in exon 2 required for their targeting to the secretory pathway. Furthermore, the splicing pattern of the 3′end of exon 3 and consequent extension of the carboxy terminal in both neuronal and immune-derived OFQ/N transcripts appears to be conserved.

Previously, a putative intronic promoter for human OFQ/N was reported to be located upstream of exon 2 (Xie et al., 1999). Since our genomic screens indicate that the intronic sequence separating exons 2 and 3 spans approximately 10 kb (unpublished observations from a genomic library screen and human genome database searches), it is unlikely that the previously described promoter region could also drive the expression of the B cell transcripts. We, therefore, postulate that a novel transcriptional start site must exist in the intronic sequence between exons 2 and 3. The analysis of 1000 bp upstream of the novel immune-derived exon (ImEx2b) with various promoter search engines yielded several putative regulation factors such as AP-1, Elk-1, Sp-1 and TEF-2 binding sites. The TEF-2 binding site is of particular interest as it has been implicated in B cell specific expression of major histocompatibility complex (MHC) class II (Ghosh et al., 1999).

In summary, this report demonstrates that upon mitogen activation, human PBLs are capable of synthesizing OFQ/N transcripts analogous to the neuronal messages. These transcripts could be translated to yield preproOFQ/N precursor subject to proteolytic cleavage and release of bioactive peptides. Since NOP functional receptor expression has been demonstrated in human PBLs, it is conceivable that OFQ/N may serve as an immune modulator. In addition, we report the expression of novel immune-derived OFQ/N transcripts in B lymphocytes. Translation of these messages would yield truncated precursor proteins devoid of a signal peptide. The functional significance and regulatory pathways of these novel OFQ/N transcripts are yet to be determined.

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