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Expression of Neurotensin/Neuromedin N Precursor in Murine Mast Cells

Hyun Jong Ahn and Jeong Je Cho

Department of Microbiology, College of Medicine, Kyung Hee University, Seoul 130–701, Korea

We have cloned the mouse neurotensin/neuromedin N (NT/N) gene from the murine mast cell line Cl.MC/C57.1 for the first time. The murine NT/N cDNA clone consisted of 765 nucleotides and coded for 169 peptide residues with an N-terminal signal peptide, and the C-terminal region contained of one copy of neurotensin (NT) and one copy of neuromedin N (NN). Total of four Lys-Arg dibasic motifs were present; one each at the middle of the open reading frame, at the N-terminal of NN, at the C-terminal of NT, and between NN and NT. Amino acid sequence analysis of the mouse NT/N revealed 90% homology to that of the rat NT/N gene. NT/N is expressed in murine mast cell lines (Cl.MC/C57.1 and P815), but not in murine bone marrow-derived mast cells (BMMCs), murine macrophage cell line (RAW 264.7), nor in murine T cell line (EL-4). NT/N mRNA in Cl.MC/C57.1 is highly inducible by IgE cross-linking, phorbol myristate acetate, neurotensin, and substance P. Following the treatment of demethylating agent, 5-azacytidine (5-azaC), the NT/N gene was induced in BMMCs in response to IgE cross-linking. 5-azaC-treated BMMCs did not express the NT/N gene without additional stimuli. These findings suggested that the regulation of NT/N gene expression was dependent on the effects of not only gene methylation but also enhancer and/or repressor proteins acting on the NT/N promoter.

Key Words: Neurotensin, Mast cell, Methylation, IgE cross-linking

INTRODUCTION

Neurotensin (NT), a tridecapeptide originally isolated from bovine hypothalami, is present in the central and peripheral nervous systems (Carraway & Leeman, 1973). In the gut, NT immunoreactivity is localized in specialized enteroendocrine cells (N cell) and neurons in the mucosa, submucosa, and muscularis of animals and humans. The gene encoding NT and the structurally related hexapeptide neuromedin N (designated NT/N) are developmentally regulated in the gut with a distinctive temporal- and spacial-specific pattern (Evers et al, 1994). NT has numerous physiologic functions in the gastrointestinal tract: NT is known to facilitate fatty acid translocation (Amstrong et al, 1986), to affect gut motility and secretion (Thor & Rosell, 1986; Riegler et al, 2000), and to stimulate the growth of normal gut mucosa (Chung et al, 1992) and certain colon and pancreatic cancers (Iwase et al, 1997). NT exerts its effects by interacting with specific cell surface G protein-coupled receptors (Tanaka et al, 1990; Mazella et al, 1996).

The factors regulating induction of the NT/N gene have been examined using rat medullary thyroid carcinoma and rat pheochromocytoma cell lines (de Nadai et al, 1993). The neuronal NT gene expression affected by estrogen involves a cAMP/protein kinase A-dependent signaling mechanism (Watters et al, 1998). NT/N expression is induced in response to combined treatment with nerve growth factor (NGF), dexamethasone, lithium, and the adenylate cyclase activator forskolin. Mutational analysis of the rat NT/N promoter identified a proximal 216-bp region of the 5' flanking sequences as essential for high-

Corresponding to: Jeong Je Cho, Department of Microbiology, College of Medicine, Kyung Hee University, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Korea. (Tel) 82-2-961-0463, (Fax) 82-2-962-6189, (E-mail) jjcho@khu.ac.kr

level constitutive NT/N expression (Kislauskis et al, 1990). The primary sequences of the gene encoding NT and the structurally related hexapeptide neuromedin N (NN) in rats and humans have also been determined (Kislauskis et al, 1988; Bean et al, 1992). Mast cells derive from a distinct bone marrow precursor and mature in tissues under the influence of stem cell factor, NGF, and certain interleukins (Metcalfe et al, 1997). They are widely distributed in the gut submucosa and are assumed to interact with the peripheral nervous system. Neuropeptides, such as NT and substance P, are secreted by the nerve stimulation and induces degranulation of mast cells (Suzuki et al, 1995; Barrocas et al, 1999). Mast cells have been reported to occur in close association with peripheral nerves in the gastrointestinal tract and at a variety of additional anatomic sites in humans and other mammalian species (Bienenstock et al, 1993). Several lines of evidence suggest the interaction between nerve cells and mast cells via neuropeptides. It has also been suggested that NT may participate in inflammatory reactions (Carraway et al, 1982; Goldman et al, 1983). Recently, NT receptor (NTR) is found from the rat serosal mast cells (Feldberg et al, 1998) and NT plays a key role in the pathogenesis of acute colonic inflammation and mast cell activation through the NTR1 receptor (Castagliuolo et al, 1999).

Previously, we have screened a mast cell cDNA subtraction library to detect novel inducible genes expressed in mast cells (Cho et al, 1999). We have found several genes which were not reported previously to be expressed in mast cells. One of the genes was of particular interest due to its significant homology with the rat and human NT/N sequences. In the present study, we have cloned the full-length mouse NT/N cDNA and analyzed the murine mast cell line Cl.MC/C57.1 and murine bone marrow-derived mast cells (BMMCs) to further clarify the mechanisms for NT/N expression. The murine NT/N sequence might be an essential element to determine the in vitro and in vivo function of this gene.

METHODS

Cell cultures

Culture of several murine cell lines: Cells of the IL-3-independently cloned murine mast cell line Cl.MC/C57.1 were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% heat inactivated fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin (complete IMDM) in a humidified 5% CO_2 incubator. P815 (murine mast cell line), RAW 264.7 (murine macrophage cell line), and EL-4 (murine T cell line) cells were maintained in RPMI 1640 with FBS and appropriate antibiotics.

Culture of murine bone marrow-derived mast cells (BMMCs): Primary culture of IL-3-dependent BMMCs was prepared from 8 to 12 week-old C57 BL/6 mice. The mice were killed and bone marrow was flushed aseptically from femurs and tibias into the IMDM medium containing 10% heat-inactivated FBS, antibiotics, and 15% (vol./vol.) WEHI 3B conditioned media as a source for IL-3. The nonadherent bone marrow cells were then maintained at 37°C at a density of 2 to 5×10^5 cells/ml in the same media, with biweekly replacement of old media with fresh ones. After 4 weeks of incubation, BMMCs were obtained, and the following experiments were performed between the sixth and tenth weeks of incubation.

Stimulation conditions

The chemical used were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise. For FcRI-dependent activation, Cl.MC/C57.1 cells were cultured to a density of 1×10^6 cells/ml in the complete IMDM containing 0.5 μg/ml of anti-dinitrophenyl monoclonal IgE at 37°C for 1 h in an incubator with gentle rocking. Cells were then centrifuged at 400 × g at 4°C for 10 min and the cell pellets were resuspended in fresh media. Phorbol myristate acetate (PMA) was added at a concentration of 50 ng/ml and calcium ionophore A23187 at 0.5 μM. Neurotensin and substance P were added at a final concentration of 100 nM. BMMCs were treated with the demethylating agent, 5-azacytidine (5-azaC), at a concentration of 4 or 8 μM and harvested 4 days after. The BMMCs pretreated with 5-AzaC were then subjected to IgE cross-linking for 3 h.

Cloning of neurotensin/neuromedin N (NT/N) gene

Since the isolated cDNA fragment containing the full coding region was not found in the subtraction library, we searched the missing farts from the EST database and found two clones matched perfectly to our sequences (accession numbers AA471944 and AA184803). Because these clones were incomplete,

the gene specific primers were prepared using sequences derived from the above clones. Amplification was conducted using the mouse mast cell activation cDNA library as the template. The PCR product was then directly cloned to pCR2.1 Topo vector (Invitrogen, San Diego, CA, USA) and sequenced by Sanger's dideoxy method using a T7 sequenase kit (Amersham Life Science Inc., Cleveland, OH, USA). The sequences were submitted to the GenBank database (accession number AF304160). The sequences of the gene-specific primer were as follows: forward, 5' gttcggatccaacgaggaagcaccga 3'/ reverse, 5' gcatccaatttacatgaatgcaag 3'.

RT-PCR

Total RNA was isolated using the TRIzol reagent (GibcoBRL, Grand Island, NY, USA). Two μg of RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase (MMLV RT) and oligo-dT primer in a total volume of 20 μl at 42°C for 1 h. The amplification process for the cDNA consisted of denaturation at 94°C for 3 min, followed by 32 cycles of incubation at 94°C for 30 sec, at 60°C for 30 sec, and at 72°C for 1 min, and a 5-min extension period at 72°C. The PCR reaction consisted of amplification of the murine NT/N cDNA of 507 bp fragment with the NT-F (5' atgagaggaatgaatctccagctg 3') and NT-R (5' gtagtagtaggaacccctcttgag 3') primers. Amplification of a fragment of the cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (324 bp) was performed over 22 cycles in the same PCR reaction which served as the internal control with the following primer sequences: forward, 5' atcaccatcttccaggagcg 3'/ reverse, 5' gatggcatggactgtggtca 3'.

RESULTS

Cloning and sequence analysis of mouse NT/N precursor cDNA

During the course of screening a mast cell subtractive library, we have previously shown that the murine homolog of the NT/N precursor was ex-

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GGCACGAGGCCAGCTGAAGGCAAGAGGAAGCACCGAGAGAGAGCTAGCTCCTTCAGTGTCTGAGTGTGTGGACCTGCTT
GTCAGAAGGCTGAGAGAAG
ATG AGA GGA ATG AAT CTC CAG CTG GTG TGC CTG ACT CTC CTG GCT TTC AGC TCC TGG AGT
M  R  G  M  N  L  Q  L  V  C  L  T  L  L  A  F  S  S  W  S
CTG TGC TCA GAT TCA GAA GAA GAT GTG AGA GCC CTG GAG GCA GAT CTA TTG ACA AAC ATG
L  C  S  D  S  E  E  D  V  R  A  L  E  A  D  L  L  T  N  M
CAT ACA TCC AAG ATC AGC AAA GCA AGT CCT CCG TCT TGG AAA ATG ACC TTG CTA AAT GTT
H  T  S  K  I  S  K  A  S  P  P  S  W  K  M  T  L  L  N  V
TGC AGC CTC ATA AAT AAC GTG AAC AGC CCG GCC GAG GAA GCA GGA GAC ATG CAT GAT GAC
C  S  L  I  N  N  V  N  S  P  A  E  E  A  G  D  M  H  D  D
GAC CTT GTT GGC AAA AGG AAA CTT CCC CTT GTT CTG GAT GGT TTT AGC TTG GAA GCA ATG
D  L  V  G  K  R  K  L  P  L  V  L  D  G  F  S  L  E  A  M
CTG ACC ATC TTC CAG CTC CAG AAA ATC TGC CGC AGC AGG GCC TTT CAA CAC TGG GAG ATA
L  T  I  F  Q  L  Q  K  I  C  R  S  R  A  F  Q  H  W  E  I
ATC CAG GAA GAT ATC CTT GAT AAC GTC AAT GAT AAA AAC GAG AAG GAA GAA GTG ATA AAG
I  Q  E  D  I  L  D  N  V  N  D  K  N  E  K  E  E  V  I  K
AGA AAA ATC CCT TAT ATT CTG AAA AGG CAG CTG TAT GAG AAT AAA CCC AGA AGG CCC TAC
R  K  I  P  Y  I  L  K  R  Q  L  Y  E  N  K  P  R  R  P  Y
ATT CTC AAG AGG GGT TCC TAC TAC TAC TGA
I  L  K  R  G  S  Y  Y  Y  stop
GAAGCTAATTCTTGACCTGTGATTGTGATTGATTACCTGATATCTAGATATATAATTATATGTGTGAATAAATGTGACA
GAACTTGATTATTTCATCTTTTCCCACAATTGTGCTTTATTGGATGTGATGTGTCTTGCATTCATGTAAATTGGATGC
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Fig. 1. Complete nucleotide sequence of the murine neurotensin/neuromedin N precursor mRNA with the amino acid sequence of the open reading frame. Detection and translation of the open reading frame were completed using the DNAsis program. Shaded box shows dibasic motifs and open box shows active forms of neuromedin N and neurotensin.

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mouse   1  MRGMNLQLVCLTLLAFSSWSLCSDSEEDVRALEADLLTNMHTSKISKASPPSWKMTLLNVCSLIN
rat     1  MIGMNLQLVCLTLLAFSSWSLCSDSEEDVRALEADLLTNMHASKVSKGSPPSWKMTLLNVCSLIN
bovine  1  MAGMKIQLVCMILLAFSSWSLCSDSEEEMKALETDLLTNMHTSKISKASVPSWKMTLLNVCSLIN
human   1  MMAGMKIQLVCMLLLAFSSWSLCSDSEEEMKALEADFLTNMHTSKISKAHVPSWKMTLLNVCSLVN
canine  1  MMAGMKIQLVCMILLAFSSWSLCSDSEEEMKALEADLLTNMHTSKISKASVPSWKMTLLNVCSFVN

mouse  66  NVNSPAEEAGDMHDDDLVGKRKLPLVLDGFSLEAMLTIFQLQKICRSRAFQHWEIIQEDILDNVN
rat    66  NLNSAAEEAGEMRDDDLVAKRKLPLVLDDFSLEALLTVFQLQKICRSRAFQHWEIIQEDILDHGN
bovine 66  NLNSQAEETGEFHEEELITRRKFPAALDGFSLEAMLTIYQLQKICHSRAFQHWELIQEDILDAGN
human  67  NLNSPAEETGEVHEEELVARRKLPTALDGFSLEAMLTIYQLHKICHSRAFQHWELIQEDILDTGN
canine 67  NLNSQAEETGEFREEELITRRKFPTALDGFSLEAMLTIYQLQKICHSRAFQQWELIQEDVLDAGN

mouse  131 DKNEKEEVIKRKIPYILKRQ LYENKPRRPYILKRGSYYY
rat    131 EKTEKEEVIKRKIPYILKRQ LYENKPRRPYILKRASYYY
bovine 131 DKNEKEEVIKRKIPYILKRQ LYENKPRRPYILKRGSYYY
human  132 DKNGKEEVIKRKIPYILKRQ LYENKPRRPYILKRDSYYY
canine 132 DKNEKEEVIKRKIPYILKRQ LYENKPRRPYILKRGSYYY
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Fig. 2. Comparison of the complete amino acid sequences of the mouse neurotensin /neuromedin N precursor with those of the rat, cow, human, and dog.

pressed at high levels in the murine mast cell line, Cl.MC/C57.1, after IgE cross-linking. To obtain the full-length cDNA, we performed PCR cloning using the mast cell activation library as a template. The murine NT/N cDNA clone consisted of 765 nucleotides. An open reading frame of this gene coded for a 169-residue polypeptide starting from an N-terminal signal peptide and contained in the C-terminal region one copy of each NT and NN. NN preceded NT and separated by a Lys-Arg (K-R) sequence. Four K-R dibasic motifs, representing the putative processing sites in the precursor molecule, are located as follows; at the middle of the open reading frame, at the N-terminal of NN, at the C-terminal of NT, and between NN and NT (Fig. 1). Amino acid sequence analysis of mouse NT/N revealed 90% homology to the rat NT/N gene (Fig. 2).

Expression of NT/N in different cell types

To determine whether NT/N is inducible, RT-PCR was performed in murine mast cell, T cell, and macrophage cell types after IgE cross-linking or stimulation with PMA/ionophore A23187. As seen in Fig. 3, murine NT/N was present only in the mast cell line. The NT/N gene was expressed at low levels in cultures of mast cell lines, Cl.MC/C57.1 and P815. Its expression level was significantly enhanced after activation with PMA/ionophore. BMMCs and other murine cell lines did not express NT/N regardless of activation.

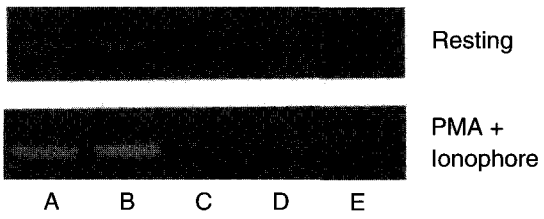

Fig. 3. Expression levels of NT in several murine cell lines before and after stimulation with PMA and ionophore. A: Cl.MC/C57.1 mast cell line, B: P815 mast cell line, C: RAW264.7 macrophage cell line, D: EL-4 T cell line, E: BMMCs.

Effects of neurotensin, substance P, PMA and IgE cross-linking on the expression of NT/N

Because neurotensin, substance P and IgE cross-linking are known to evoke mast cell activation and degranulation, Cl.MC/C57.1 cells were treated with these reagents to observe the effects on NT expression. Upon stimulation with neurotensin itself, NT mRNA was rapidly up-regulated and detectable as early as 30 min after stimulation. Substance P induced NT expression 1 h after stimulation. Induction of NT expression by PMA was detectable within 30 min

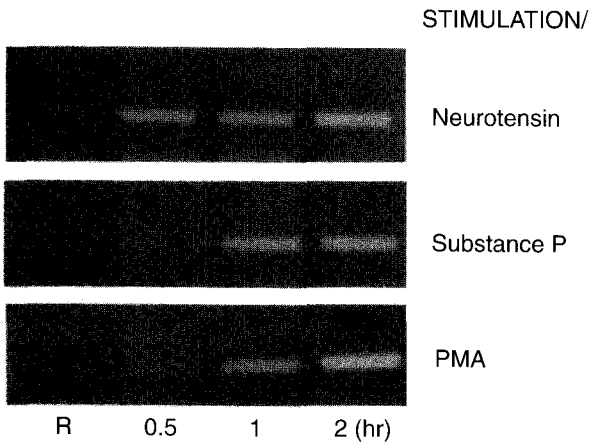

Fig. 4. Effects of neurotensin, substance P, and PMA on the expression of NT in Cl.MC/C57.1 cells at different time points. R: control.

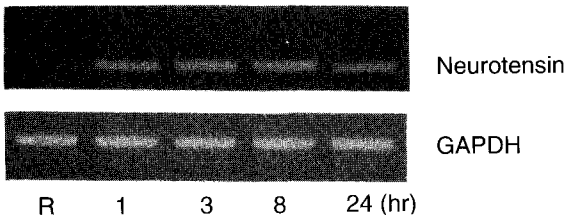

Fig. 5. Time-wise expression pattern of NT in Cl.MC/C57.1 cells after IgE cross-linking. R: control.

with increasing intensities in a time-dependent manner (Fig. 4). IgE cross-linking induced NT mRNA expression, reaching maximum expression levels within 1 h, but the intensity was not further changed after longer periods of stimulation (Fig. 5).

NT/N gene suppression in BMMCs is mediated by gene methylation

The role of methylation in regulating NT/N gene expression was assessed by treating BMMCs with 5-azaC. Cells treated with 4 or 8 μM 5-azaC for 4 days demonstrated a high level of NT/N expression after IgE cross-linking. Incubation with 8 μM 5-azaC for 4 days resulted in a similar intensity of NT/N expression. However, even with 5-azaC treatment, BMMCs did not express the NT/N gene without IgE cross-linking (Fig. 6).

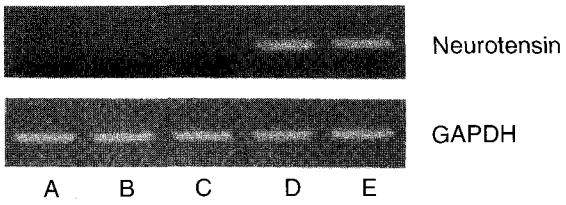

Fig. 6. Effects of the demethylating agent 5-azacytidine on the expression of NT in BMMCs. A: No treatment, B: Ethanol treatment, C: 5-azacytidine (4 μM), D: 5-azacytidine (4 μM) and IgE cross-linking, E: 5-azacytidine (8 μM) and IgE cross-linking.

DISCUSSION

The present study reports the coding region of mouse NT/N gene and its expression pattern for the first time from mast cells. Sequence analysis of the mouse NT/N gene revealed that the four dibasic motifs were present in the same positions as they are in the rat gene. These dibasic motifs are necessary and play a role to signal for sorting proteins and regulate secretory pathway (Feliciangeli et al, 2000). The processing of the NT/N precursors is thought to occur in the regulated secretory pathway where it is initiated by cleavage at the C-terminal sides of the dibasic residues by prohormone convertases, followed by carboxypeptidase E-catalyzed removal of C-terminal basic residues (Steiner et al, 1992; Halban et al, 1994), although presence of these enzymes in mast cells has not been confirmed as yet.

In the present study, we have demonstrated constitutive expression of the terminally differentiated endocrine gene NT/N in the murine mast cell line Cl.MC/C57.1 but not in BMMCs, the murine T cell line EL-4, and the murine macrophage cell line RAW264.7. We have also found that NT/N expression is highly inducible after IgE cross-linking and PMA/ionophore treatment of Cl.MC/C57.1 and P815 cells, respectively. In the promoter analysis using the BON cell line, CRE/AP-1 like motif, near-consensus glucocorticoid response element and 21-bp imperfect direct repeat conducted as positive transcriptional regulators (Evers et al, 1995). Transactivation of AP-1 is considered to be required for PMA-stimulated cellular proliferation and transformation (Angel et al, 1991). Therefore, PMA or IgE cross-linking may act on the NT/N promoter via the protein kinase C path-

way.

Gene methylation appears to be involved in several crucial cellular processes, including differentiation, development, and carcinogenesis (Cedar & Razin, 1990; Kafri et al, 1992; Lengauer et al, 1997). The expression of NT/N gene in HepG2 cells after treatment of 5-azaC suggests that DNA methylation plays a role to suppress NT/N gene in HepG2 cells (Dong et al, 1998). The results from the present study suggest that DNA methylation plays a role in NT/N gene regulation in mast cells. After treatment with a demethylating agent, the NT/N gene was induced in BMMCs in response to IgE cross-linking. Because 5-azaC-treated BMMCs do not express NT/N gene in the absence of additional stimuli, gene methylation alone does not fully account for the marked suppression of NT/N in BMMCs. Therefore, the strict tissue- or cell-specific regulation of NT/N gene expression appears to be dependent on the combinatorial effects of not only gene methylation, but also of enhancer and/or repressor proteins that bind to the NT/N promoter.

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