Regulation of Mu Opioid Receptor Gene Transcription by Interleukin-4 and Influence of an Allelic Variation Within a STAT6 Transcription Factor Binding Site

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Running title: Regulation of Mu Opioid Receptor Gene Transcription by Interleukin-4
Summary
Morphine and the endogenous opioid peptide β-endorphin exert neuromodulatory as well as immunomodulatory effects, which are transduced by mu opioid receptors. In this report we show that stimulation with interleukin-4 induces mu opioid receptor transcripts in human primary blood cells (T-cells, polymorphonuclear leukocytes), immune cell lines (Raji, U-937, HMEC-1) and dendritic cells. In non-stimulated immune cells this gene is silent. In addition, mu receptor transcription is upregulated by interleukin-4 in cultures of primary rat neurons. Transient transfection experiments in Raji and SH SY5Y neuronal cells with human and rat reporter gene constructs linked the interleukin-4 effect directly to cis-active mu receptor promoter elements located at nucleotide -997 on the human gene and nucleotide -727 on the rat gene. The interleukin-4 response elements function orientation independently. They bind STAT6 transcription factors, as shown by electrophoretic mobility shift assays. In the human gene, a single nucleotide polymorphism within the interleukin-4 response element reduces the trans-activating potential of this element by 50%, which may affect the phenotype of persons carrying this variation. These findings provide a molecular basis for understanding bidirectional interactions between the opioid system and the immune system.

Introduction
Opioids are classically associated with phenomena such as analgesia, respiratory depression and addiction. The effects of opioids are mediated by at least three different opioid receptors, termed mu, delta and kappa, which belong to the G-protein coupled membrane receptor family (1,2). During the last years, evidence accumulated showing that exogenous opiates like morphine and endogenous opioid peptides derived from the precursors proopiomelanocortin, proenkephalin and prodynorphin have multiple immunomodulatory properties, in addition to their classical functions as neuromodulators. It was stated that the endogenous opioid peptides would now be considered members of the cytokine family, if they had been first discovered by immunologists (3). Elucidation of a broad bidirectional communication between the opioid system and the immune system strengthens this concept. Thus, morphine, the prototypical
exogenous ligand for the mu receptor, is an immunosuppressive drug and responsible for increased susceptibility of opioid addicts to infections (4-8). Studies with mu opioid receptor knock out mice emphasised the role of this receptor in immunosuppression (9). After chronic morphine treatment, these mice developed none of the symptoms characteristic for wild type mice, namely lymphoid organ atrophy, diminished CD4(+)CD8(+) cell ratio and strongly reduced natural killer cell activity. Vice versa, cytokines modulate the expression of opioid peptide genes and opioid receptor genes. For example, studies with IL-6* knock out mice revealed decreased levels of mu receptors in the brain compared to wild-type animals, suggesting a positive regulation of this receptor by IL-6 in vivo (10). In vitro, up-regulation of mu receptors was observed after IL-1 stimulation in primary astrocyte-enriched cultures derived from various brain regions (11,12) and in neural microvascular endothelial cells (13). In promoter studies, however, a direct activation of mu receptor transcription by IL-1 and IL-6 could not been demonstrated (14).

The opioid peptide β-endorphin is derived from proopiomelanocortin and is a physiological endogenous ligand of the mu receptor. A close relationship was demonstrated between morphine and β-endorphin on the one hand and T cell regulation on the other. Thus, immunological effects of β-endorphin are exerted via activation of IL-4, the prototypical Th2 cytokine (15,16). In addition, morphine as well as β-endorphin were shown to induce a shift from Th1 type to Th2 type cytokine pattern (8,17). Vice versa, blocking of β-endorphin by antibodies or blocking of mu opioid receptors by the highly specific mu receptor antagonist naloxone resulted in a shift from Th2 type to Th1 type cytokine pattern (18,19).

Considering the apparent involvement of mu opioid receptors in immunosuppression and immunomodulation, and the multiple regulatory interactions between cytokines (in particular Th2 cytokines) and the mu opioid system, we addressed the question whether IL-4 may regulate expression of the mu opioid receptor gene and investigated the molecular mechanisms of this regulation.

**Experimental Procedures**
Cell culture

SH SY5Y and HEK 293 cells were cultivated in DMEM (PAA Laboratories, Linz, Austria) supplemented with 15% FCS (PAA Laboratories, Linz, Austria) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). HMEC-1 cells were kept in endothelial growth medium EMG-2 (Clonetics, San Diego, CA, USA) with the growth factors and supplements suggested and supplied together with the medium by the manufacturer. Raji and U-937 cells were cultivated in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mM N-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany), 10 % FCS and antibiotics. Human primary T cells and PMN were separated from blood as described (20) and kept in RPMI 1640 medium with 1% FCS.

Dendritic cell preparation

Peripheral primary human monocytes were isolated from healthy donors and differentiated into dendritic cells according to described protocols with GM-CSF (800 U/ml) and IL-4 (days 0-2: 1000 U/ml, days 2-9: 500 U/ml) (21-23). On the ninth day of differentiation FACS® analysis was performed with FACscalibur (Becton-Dickinson, Heidelberg, Germany). Cells were double stained with PE-labeled CD14 and FITC-labeled CD83 antibodies (Becton-Dickinson, Heidelberg, Germany). and checked for expression of CD83 (>80%) versus CD14 (<10%).

Preparation of primary cortical neurons

Fetal cortical neurons were prepared as described (24) with the following modifications. In brief, 18-19 day old fetal rats were removed from the uterus followed by immediate preparation of their brains. The cerebral cortices were dissected and transferred to DMEM. The tissue was disassembled into single neurons using a syringe equipped with a 21G x 1.5 needle. The cells were washed twice with DMEM and plated in six well tissue dishes coated with Matrigel (Beckton Dickinson, Heidelberg, Germany). The cells were kept for 24 h in DMEM containing 10% FCS. The cells were further cultivated for four days in Start V medium (Biochrom, Berlin, Germany) without FCS, then stimulated and harvested for RNA extraction.
Cytokines
Twenty-four h prior to cytokine stimulation all cells including controls were given fresh medium with only 1% FCS to minimize interference with substances present in the serum. In all experiments, recombinant human IL-4 (R&D Systems, Wiesbaden, Germany) was used in a final concentration of 5 ng/ml, which equals approximately 150 WHO U/ml for stimulation of human cells, except for differentiation of dendritic cells (see there for different concentrations). Rat cell cultures were stimulated with 5 ng/ml recombinant rat IL-4 (R&D Systems). Stock solutions of 5 µg/ml in PBS buffer with 0.5 % BSA were kept at -20°C in frozen aliquots to avoid repeated freezing and thawing. Human recombinant GM-CSF was purchased from R&D Systems.

Reverse transcription (RT)-PCR from human immune effector cells
Total RNAs from immune cells were extracted using RNeasy columns (Qiagen, Hilden, Germany) and then checked for accidental degradation on an agarose gel. Two µg total RNA were used for cDNA synthesis with MMLV reverse transcriptase, RNase H minus (Promega, Mannheim, Germany) and diluted to 100 µl. To check the relative amount and the integrity 2 to 4 µl cDNA were used to amplify a 842 bp fragment of the human GAPDH gene (forward primer 5’-CAACTACATGGTTTACATGTTC-3’, reverse primer 5’-TGACAACGAATTTGGCTACAG-3’) under the following conditions: 25 cycles with 40 s at 94°, 60 s at 60° and 180 s at 72°. Two consecutive amplification steps were performed to amplify mu opioid receptor transcripts: a first PCR reaction with 2-5 µl cDNA (adjusted amounts that gave similar intense bands in a GAPDH-PCR) in a 30 µl volume with the forward primer 5’-CTTGGCGTACTCAAGTTGCT-3’ and the reverse primer 5’-AATGTGAATGGGAGTCCAGC-3’ for 25 cycles of 40 s at 94°, 60 s at 63° and 90 s at 72° with an initial denaturation step (150 s at 94°) and a final prolongation step (120 s at 72°). Five µl of the first reaction were used for a second PCR reaction in a 30 µl volume with the forward primer 5’-GATCATGGCCCTCTACTCCA-3’ and the reverse primer 5’-GCATTTCGGGGAGTACGGAA-3’ for 30 cycles of 40 s at 94°, 60 s at 65° and 90 s at 72° with an initial denaturation step (150 s at 94°) and a final prolongation step (120 s at 72°).
both reactions the forward and reverse primers are located on different exons to ascertain that no genomic DNA amplification may mask the cDNA specific amplificate. Five µl of the second PCR reaction were electrophorized on a 1.5% agarose gel.

Real time RT-PCR from rat neuronal cells
Total RNA was extracted from primary neurons using TRI-Reagent (Sigma-Aldrich, Germany) following the manufacturer’s instructions. The RNA was checked on an ethidium bromide stained agarose gel for integrity and to ensure equal amounts per sample. Four µg of each RNA sample were subjected to reverse transcription using TrueScript MMLV reverse transcriptase (Hybaid, Heidelberg, Germany) in a total volume of 20 µl. After incubation at 42° for 1 h the reaction mixture was diluted to 100 µl and 2 µl were included in the PCR reaction (Light Cycler - Fast Start DNA Master SYBR Green I kit, Roche, Mannheim, Germany) in a total volume of 20 µl. The primers used to detect rat mu opioid receptor transcript were 5’-CATTACCATCATGGCCCTCTACTCTATCG-3’ and 5’-ACTGGGTGGCAGACAGCAATGTAGC-3’ yielding an amplified product of 315 bp. The primers spanned an intron to avoid amplification of genomic DNA. The reaction was run in a Light Cycler PCR machine (Roche, Mannheim, Germany) with a hot start preincubation step of 10 min at 95° for 50 cycles consisting of denaturation (15 s, 95°) and annealing/elongation (30 s, 72°). To obtain a standard curve, the stimulated samples were pooled and serially diluted. The amount of template was quantified using the second derivative maximum method as included in the Light Cycler software package.

Construction of reporter plasmids
All reporter plasmids are based on the pBLCAT2/pBLCAT3 vector system (25). The series of rat mu promoter reporter plasmids had been used and described earlier (26). For construction of plasmid prMOR-1660Δ-851/-449 the basic -1660 construct was opened with SpeI (nt - 617) and digested with Bal31. The enzyme was inactivated by 20 min at 80°C, the ends were blunted with Klenow enzyme and religated. This and all other plasmids used here were sequenced - if possible from both sides - to ensure correct cloning procedures and
determination of the extend of Bal31 digests. The basic human mu promoter reporter plasmid (phMOR-2229) was constructed as follows: starting from previously cloned sequences (27) a SauI - BglI fragment spanning from nt -2229 to nt -165 containing the prominent transcription initiation sites was inserted into the BamHI and BglII sites of the vector pBLCAT2 (25). Simultaneously, the HSV tk promoter located between these vector sites was excised. Construct phMOR-1372 was generated by cutting the basic construct with BglII (nt -1372) and HindIII (vector), filling the ends and religating. Construct phMOR-779 was obtained by a 5’ deletion method using DNase as described (28). Constructs phMOR-2229Δ-1854/-1372 and phMOR-2229Δ-1372/-254 were generated by cutting out fragments using the restriction enzymes AccI (nt -1854), BglII and PstI (nt -254), blunting and ligating. The internal deletions in the plasmids phMOR-2229Δ-1933/-1033 and phMOR-1372Δ-1001/-950 were made with enzyme Bal31 after opening the plasmids with BglII and NdeI (nt -985), respectively. Oligonucleotides with IL-4 responsive regions were inserted in pBLCAT2 in front of the HSV tk promoter into the vector’s XbaI (human constructs) or SphI (rat construct) sites. The sequences of the inserted human -997 wild type, human -997 polymorphic and rat -727 oligonucleotides are given in this section under "Electrophoretic mobility shift assay", since the same double stranded oligonucleotides were used.

Transfection experiments

The plasmid DNA used for transfection was isolated using Qiagen Plasmid Kits (Qiagen, Hilden, Germany). Before transfection, all cells received fresh medium with 1% FCS. Transfection of SH SY5Y cells and reporter gene assay have been described earlier (26,29). The same method was applied to transfect HMEC-1 and HEK 293 cells. Raji and U-937 cells were transfected as follows: Five times 10^6 cells were pelleted, resuspended in 5 ml medium and transfected by dropwise addition of 10 μg plasmid DNA in 500 μl of a buffer containing 140 mM NaCl, 25 mM Hepes and 0.5 mM Na2HPO4 (pH 7.05). After a 17 h incubation at 3% CO2 and 35° C, cells were pelleted again and given fresh medium. After transfection, all cells were allowed to grow for further 48 hrs in a 1% FCS medium containing IL-4 or vehicle prior to the CAT ELISA (Roche, Mannheim, Germany).
Extraction of nuclear protein

Twenty-four hours prior to nuclear protein extraction SH SY5Y cells received fresh medium containing 1% FCS. Stimulation with 5 ng/ml IL-4 was for 1 hour prior to nuclear protein extraction. The extraction procedure for nuclear proteins used in electrophoretic mobility shift assays has been described in detail in a recent publication (30).

Electrophoretic mobility shift assay (EMSA)

Synthetic double stranded oligonucleotides (Metabion, Martinsried, Germany) carrying putative STAT6 binding sites of mu opioid receptor promoters and the IL-4-response element of the immunoglobulin heavy chain germline ε promoter ("Classic STAT6") (31) were labeled with [\(\gamma\)-32P]ATP (Amersham, Braunschweig, Germany) according to standard methods (32). For each band shift reaction 5000 cpm of labeled probe were incubated with 3 µl SH SY5Y cell nuclear extract for 15 minutes at room temperature in a 20 µl reaction mixture containing 10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.1 µg poly(dIdC) DNA. Some reactions contained additionally various amounts of competitor DNA. For immunoshift experiments 4 µg STAT6 antibody ("M-20", Santa Cruz Biotechnology, Heidelberg, Germany) were added after the reaction and incubated for further 60 min. Samples were loaded onto 6% high-ionic-strength polyacrylamide gels with the Tris-glycine buffer system and separated electrophoretically. The gels were then dried, exposed to BAS1000 imaging plates and analysed by the BAS1000 phosphoimaging program. Sequences of the oligonucleotides were: AP-2 motif used as unspecific competitor: 5'-TGC GGCTCCCCGGCGGTGGCGAGC-3'. Classic STAT6: 5'-TAGTCAACTTCCCAAGAAGAGCAGC-3'. Human -997 motif (wild type): 5'-CAACCTTCTTTCAGAAGCATATGT-3'. Human -997 motif (polymorphism): 5'-CAACCTTCTTTCAGAAGCATATGT-3'. Rat -727 motif: 5'-TGT TTCCTTTCCAGAAGGACCATT-3'.

Statistical analysis

For statistical evaluation of the transfection experiments Student’s t-tests were performed.
Results

Induction of mu opioid receptor transcripts by IL-4 in immune effector cells.

For a first set of experiments (fig. 1A) primary human T cells and PMN, and in addition, the human cell lines Raji (B cells), U937 (monocytes) and HMEC-1 (microvascular endothelial cells) were used to study the effect of IL-4 on the expression of mu opioid receptors in various immune effector cells. The human neuronal cell line SH SY5Y (neuroblastoma), which is known to express mu opioid receptors constitutively (33), served as positive control (lane 11). No transcripts of mu opioid receptors could be detected in any of the unstimulated immune cells (lanes 1, 3, 5, 7 and 9). The highly sensitive nested PCR technique, which was used for these experiments allowed us to detect as few as five copies of mu opioid receptor sequences (data not shown), suggesting that in unstimulated immune cells virtually no mu opioid receptor expression occurs. When the immune cells were stimulated with 5 ng/ml IL-4 for 24 h, mu opioid receptor transcription was strongly induced in all cells tested (lanes 2, 4, 6, 8 and 10).

In a second set of experiments mu opioid receptor transcription was determined in monocytes and dendritic cells (fig. 1B). Peripheral monocytes were differentiated into dendritic cells with GM-CSF and IL-4 according to described methods (21,22). After nine days differentiation, the cells were morphological mature dendritic cells with typical filopodia. FACS® analysis during maturation revealed a reduction of CD14 expression (monocyte marker) paralleled by an induction of CD83 expression (dendritic cell marker). Mu opioid receptor transcripts were detected neither in undifferentiated monocytes (lane 1), nor in cells during maturation (i. e. after four days incubation with IL-4/GM CSF, lane 2), nor in the CD83+ cells (i. e. nine days IL-4/GM-CSF, lane 5). This was unexpected, since incubation of other immune cells with IL-4 persistently induced mu opioid receptor transcription (fig. 1A). However, when monocytes were incubated with IL-4 alone, mu opioid receptor transcripts were detected (lane 3). When CD83+ dendritic cells from day nine were further incubated for 24 hours exclusively with IL-4 (i. e. after removing the GM-CSF containing medium) mu opioid receptor
transcription was also induced (lane 6). These experiments indicate that GM-CSF inhibits mu opioid receptor transcription induced by IL-4.

Regulation of mu opioid receptor transcripts in primary cortical neurons from rats.
Cultures of primary cortical fetal neurons from rats were used to investigate whether IL-4 had also a regulatory effect on mu opioid receptor transcription in cells of neuronal origin (fig. 2). In contrast to immune cells, mu receptor transcripts were already present in the neuronal cells under basal condition. Therefore, to determine quantitative changes in levels of mu opioid receptor transcripts, and the time course of induction by IL-4, the "real time PCR" technique was employed. This technique allows to monitor the onset of the exponential phase of amplification during the PCR run. The earlier this amplification phase begins, the more template is present in the reaction. The experiments showed that IL-4 induced a transient increase in mu opioid receptor transcription in the neuronal cells. About 2.7-fold increased levels of mu receptor transcripts were observed already after 30 min of IL-4 incubation. This peak was followed by decreasing levels of mu opioid receptor transcripts which dropped to control levels again, when cells were incubated for 4 h with IL-4.

Inducibility of human and rat mu opioid receptor gene promoters by IL-4.
Transient transfection experiments with mu promoter-reporter gene constructs were performed in various cell lines to test whether IL-4 regulates mu opioid receptor gene expression at the level of transcription initiation (fig. 3). Plasmid pBLCAT2 (25), which was used as a construction vector containing the HSV tk promoter instead of mu receptor promoters, showed no significant inducibility with IL-4 in all of the cells tested (fig. 3C). A basic construct with human upstream mu opioid receptor promoter sequences contained the region spanning from nt -2229 to -165 (with respect to the first translated codon; construct phMOR-2229) in front of the CAT reporter gene. A basic construct with the rat promoter included 5’ upstream sequences up to nt -1660 (construct prMOR-1660). Stimulation of transfected cells with IL-4 (5 ng/ml) significantly elevated CAT activities for both the human (fig. 3A) and the rat (fig. 3B) promoter constructs in Raji, U-937 and HMEC-1 cells approximately 3- to 4-fold. A similar
induction of mu receptor transcription was observed in neuronal SH SY5Y cells. In contrast, no induction after IL-4 treatment of transfected cells was found in HEK 293 cells. The latter cells are known to lack STAT6 transcription factors but contain other IL-4 signalling components (34). Unraveling the molecular mechanisms of gene regulation by cytokines, it was found that interleukines, interferons and growth factors induce the JAK/STAT signal transduction pathway (35,36). All STAT (=signal transducers and activators of transcription) transcription factor family members bind to very similar cis-active elements on target gene promoters with the palindromic binding motif 5’-TTCn2-4GAA-3’ (37). IL-4 regulated gene expression of target genes is transduced typically via STAT6 (37-41). The results obtained in HEK 293 cells thus suggest contribution of STAT6 to IL-4 mediated regulation of the mu opioid receptor gene.

Localization of IL-4 responsive promoter elements.
Raji and SH SY5Y cells were used for further transfection experiments with deletion constructs containing various lengths of 5’ flanking regions of the mu opioid receptor promoters in order to localize promoter elements required for IL-4 inducibility. The conclusions which can be drawn from the experiments with respect to IL-4 inducibility are identical for both Raji and SH SY5Y cells. In the following text it was therefore not discriminated between the two cell types, however, results for both cells are shown separately in the figures. The human promoter contains three motifs of the sequence 5’-TTCn2-4GAA-3’ with three central nucleotides, which may serve as binding sites for the STAT6 transcription factor, located at nt -1583, -1061 and -997 (fig. 4A). When the longest construct with all putative STAT6 binding motifs (lane 1, construct phMOR-2229) was shortened at its 5’ end deleting the most distal -1583 putative STAT6 motif, IL-4 inducibility was retained (lane 2, construct phMOR-1372). A construct with a further 5’ deletion up to nt -779, in which also the remaining two STAT6 motifs were deleted, showed no stimulation (lane 3, construct phMOR-779). Constructs with internal deletions were made to test which motif(s) mediate IL-4 stimulation of reporter gene expression. As expected from the above results, a construct in which only sequences containing the distal -1583 motif was deleted, was still responsive to
IL-4 (lane 4, construct phMOR-2229Δ-1854/-1372). A construct in which the -1061 motif was additionally deleted was also responsive to IL-4 (lane 5, construct phMOR-2229Δ-1933/-1033), indicating that the proximal motif may be involved. This was indeed true, since, deletion of the proximal motif at nt -997 abolished the IL-4 effect (lane 6, construct phMOR-2229Δ-1372/-254 and lane 7, construct phMOR-1372Δ-1001/-950). Taken together, the transfection experiments with the human promoter demonstrated that a region spanning from nt -950 to nt -1001, which contains the proximal putative STAT6 motif at nt -997, is sufficient to mediate the IL-4 stimulatory effect.

Like the human promoter, the rat mu opioid receptor promoter also contains three putative STAT6 binding sites located at nt -1353, -982 and -727 with two, three and four central nucleotides, respectively (fig. 4B). Transfection of a series of 5’ deletion constructs of the rat promoter suggests that, like in the human gene, sequences around the proximal putative STAT6 motif (nt -727) are necessary for IL-4 induction of this promoter. Thus, neither deletion of the distal motif (lane 2, construct prMOR-1198), nor additional deletion of the middle motif (lane 3, construct prMOR-974) affected IL-4 responsiveness of the reporter gene constructs. When also the proximal motif was deleted, the IL-4 effect was abolished (lane 4, construct prMOR-605). A construct with an internal deletion of sequences around the proximal motif showed no inducibility (lane 5, construct prMOR-1660Δ-851/-449). Taken together, these experiments showed that sequences of the rat promoter spanning from nt -605 to nt -851, which contain the proximal putative STAT6 motif at nt -727 are sufficient to mediate the IL-4 effect.

Oligonucleotides containing the proximal motifs were then cloned in front of the tk promoter in pBLCAT2 to test whether they are sufficient to mediate IL-4 responsiveness (fig 4C). The human -997 (lane 1) and the rat -727 (lane 3) motif both conferred IL-4 responsiveness to the heterologous tk promoter, independently of the orientation (lanes 2 and 4 show the constructs with the motifs cloned in the antisense orientation), demonstrating that these sequences are sufficient to mediate IL-4 induction of the mu opioid receptor gene.

The IL-4 responsive promoter elements bind STAT6 transcription factors.
Using the EMSA technique, the IL-4 responsive elements of the human and rat mu opioid receptor gene promoters were characterized. Incubation of an oligonucleotide probe containing the IL-4 response element of the immunoglobulin heavy chain germline ε gene promoter (termed "classic STAT6" oligonucleotide) with nuclear proteins isolated from IL-4-stimulated SH SY5Y cells resulted in a pattern similar to that shown originally by these authors (31). Of the two prominent low mobility complexes C1 and C2, C1 had been identified as the specific IL-4 inducible complex (31) (fig. 5A and B, lanes 2). A kinetic experiment showed that complex C1 was induced maximally after 30 min to 2 h of IL-4 treatment of cells prior to nuclear protein extraction (data not shown). The C1 complex, and to a lesser extend also C2, were competed by the homologous classic STAT6 oligonucleotide (A and B, lanes 4 and 5), but not unspecificly by an AP-2 oligonucleotide (A and B, lanes 3) derived from the delta opioid receptor (42). Human -997 oligonucleotides (fig. 5A, lanes 6 and 7) and also rat -727 IL-4 motif oligonucleotides (fig. 5B, lanes 6 and 7) were as effective in competition as classic STAT6 oligonucleotides themselves, indicating that the same nuclear factors bind to these sequences. Vice versa, when oligonucleotides containing the mu opioid receptor IL-4 response elements were used as probes, EMSA patterns similar to those obtained with classic STAT6 oligonucleotides were obtained (A and B, lanes 8) and the specific complexes were competed by classic STAT6 oligonucleotides (A and B, lanes 9 and 10). Supershift experiments directly demonstrated binding of this factor to mu opioid receptor IL-4 responsive elements of both species. Thus, using a specific STAT6 antibody, the IL-4-inducible complex C1 disappeared (A and B, lanes 11 and 12). To a lesser extend, also complex C2 is affected by the STAT6 antibodies, indicating that it may also contain small amounts of STAT6 protein.

A single nucleotide polymorphism in the human IL-4 responsive element decreases IL-4 inducibility.

Recently, the human mu opioid receptor gene has been studied for sequence variability among 250 individuals revealing at least 43 variants within the gene (43). More than half of these, 28, were found in the 5’ regulatory region. Interestingly, one such naturally occurring sequence variation in the promoter region leads to a single nucleotide exchange within the -997 IL-4
response element. Thus, the wild type sequence 5’-TTCTcAGAA-3’ is changed into 5’-TTaTcAGAA-3’, interrupting its palindromic structure (throughout this report, palindromic nucleotides within IL-4 response elements/STAT6 motifs are shown in upper case). Therefore, both alleles were compared to reveal if the polymorphism changes the trans-activation potential of the motif and/or binding of STAT6. Transfection of the mutated allele (fig. 6A, lanes 3 and 4) resulted in a dramatically decreased IL-4 response, compared to the human wild type sequence (fig. 6A, lanes 1 and 2). On the other hand, EMSAs comparing the wild type and the mutated motifs revealed no difference in binding to STAT6. Thus, both motifs were equally effective competitors for complex C1 using the classic STAT6 probe (fig. 6B). Additionally, in spite of their markedly different trans-activating potentials, the polymorph motif as a probe gave similar EMSA patterns as the wild type motif and supershift experiments demonstrated STAT6 binding to the mutated motif, as well (fig. 6C).

**Discussion**

Earlier studies demonstrated immunosuppressive and immunoregulatory functions of opioid alkaloids like morphine and endogenous opioid peptides such as β-endorphin (5,8,15,19). Beta-endorphin has been considered as a neuropeptide as well as a Th2 cytokine (44). In the present study we have shown that mu opioid receptors, for which β-endorphin and morphine are ligands, are induced by the Th2 cytokine IL-4 in native immune cells, immune cell lines, dendritic cells and also in primary neuronal cells. The IL-4 signal is directly transduced to the mu receptor gene promoter via STAT6.

The multiple effects of opioids on immune effector cells obtained from functional studies implicate that opioid receptors are expressed on these cells (4,9,19,45,46). However, data obtained from different laboratories are rather contradictory when mu receptor transcripts were attempted to be demonstrated directly. Thus, mu opioid receptor transcripts were detected in lymphocytes, monocytes and macrophages by some researchers (47,48), whereas other groups failed to detect mu receptor expression, but consistently showed delta and kappa opioid receptor expression in immune cells (49,50). Our experiments showed that mu opioid receptor
expression in immune effector cells strongly depends on cytokine stimulation. Thus, variations in cytokine concentrations present in cell culture medium may be an explanation for different results by different groups. The results of the experiments with dendritic cells suggested that other cytokines may also regulate mu receptor expression, since the IL-4 effect was inhibited by GM-CSF. Regulation of the mu opioid receptor gene by other cytokines is currently investigated and will be focussed in future communications.

Transcriptional effects of cytokines on selected genes often occur indirectly, because of cascade-like induction of secondary cytokines. Direct transcriptional activation of target genes by IL-4 is transduced typically via the transcription factor STAT6 (38). The IL-4 responsive elements of the human and rat mu opioid receptor gene promoters are STAT6 binding sites. The human element contains three central nucleotides ("N3 motif") and the rat element four ("N4 motif") between the two halves of the palindrome 5’-TTCn2-4GAA-3’ . Both element have, independent of their orientation, similar strong trans-activating potentials as demonstrated by experiments with the heterologous HSV tk promoter. A single nucleotide exchange within the -997 IL-4 response element, which occurs as a genetic variation in humans, changes the sequence 5’-TTC...GAA-3’ into 5’-TTa...gAA-3’ (43). Although binding of STAT6 to the mutated sequence is not notably different compared to STAT6 binding to the wild type motif, the trans-activating potential of this sequence is markedly reduced to about 50% compared to the wild type. The following observations may help to explain this discrepancy in spite of our limited knowledge about the mechanisms of STAT6-mediated transcriptional activation. On the one hand, in vitro studies using a variety of DNA probes that contain the 5’-TTCn2-4GAA-3’ motif together with randomly chosen nucleotides in the center and outside the palindrome revealed that N4 motifs bind STAT6 with higher affinity than N3 motifs (34,39). On the other hand, it was demonstrated in the same studies, that the same N4 motifs that bound STAT6, were not responsive to IL-4 in transfection experiments (34,39). Thus, binding of STAT6 to a 5’-TTCn2-4GAA-3’ motif does not necessarily mean that this motif has also trans-activating potential in response to IL-4. Vice versa, it may be not astonishing that the mutated human -997 element binds STAT6 with similar affinity as the wild type element in spite of a reduced trans-activating potency.
Moreover, binding of other STAT proteins to the palindromic sequence consisting only of 5'-TTn4-6AA-3' also occurs in other genes responding to cytokines as listed by Decker and colleagues (37). IL-4/STAT6 induced transcription may require the interaction between multiple transcription factors, as demonstrated for the murine germline ε gene promoter, where a N4 STAT6 motif is adjacent to a C/EBP motif and both factors are necessary for transcriptional activation from this promoter (31). Predicted from their sequences, mu opioid receptor promoter sequences that confer IL-4 induction are unlikely to bind other transcription factors. Alternatively, co-activators of transcription which mediate between STAT6 and the general transcriptional machinery may be necessary, similar to CREB induced transcription mediated via the CREB binding protein CBP (51). Among naturally occurring IL-4 responsive sequences, the motif of the low affinity IgE receptor (CD23b) is a N3 motif in which the palindromic halves are extended: 5'-TTTCTaAGAAA-3' (52). Interestingly, the IL-4 response elements of the mu opioid receptors from both species are also symmetrically extended, whereas all the other 5'-TTCn2-4GAA-3' motifs of these genes, which were not involved in IL-4 regulation, consisted only of the minimal palindrome. The human IL-4 responsive element is 5'-CTTCTcAGAAG-3' and the rat motif is 5'-CTTTCcagaGAAAG-3'. This observation suggests a possible functional importance of palindromic nucleotides immediately adjacent to the 5'-TTC-3' and 5'-GAA-3' sequences for trans-activation. In future, identification of additional response elements on other IL-4 responsive genes and comparison of their sequences will be required to reveal a more precise structure-function relationship.

A challenge in the "post-genomic era" will be elucidation of functional allelic variations with defined molecular effects. Among the variants of the human mu opioid receptor gene, those found in the 5' regulatory region may be of remarkable functional importance if they affect transcriptionally regulatory motifs as demonstrated here for IL-4 response elements. It will be interesting to correlate this polymorphism to a certain phenotype. To date, however, the number of individuals known to carry this variation is too low for such studies (43). With respect to genes of the opioid system, we earlier reported on a functional polymorphism within the prodynorphin gene promoter, which consists of a repeat containing an AP-1 transcription factor site. The number of this repeat varies among individuals and so does the promoter’s
response to stimuli of AP-1 (53).

IL-4 may also be involved in the regulation of mu opioid receptor expression in neuronal cells. Increasing information about IL-4 and its receptors in brain suggests the importance of this cytokine in neuronal environment (54,55). It was shown that high levels of IL-4 are present in brains of neonatal mice (56). It may be interesting in this context that high levels of STAT6 were also found in developing brains and that they were localized to regions that abundantly express mu receptors, namely hippocampus, striatum and cortex (57), suggesting a possible role of IL-4 in developmental regulation of mu receptor expression. In general, it should be considered that changes in IL-4 concentrations within the central nervous system may potentially affect the effectivity of analgesic drugs by modulating the number of mu opioid receptors. Recently, changes in IL-4 and/or IL-4 receptor levels associated with distinct cerebral diseases such as Parkinson's disease (58) and brain ischemia (59) were demonstrated. Likewise, the approved drug copolymer 1 which is used for treatment of multiple sclerosis exerts its effects via activation of Th2 cytokines including IL-4 (60). Knowledge about regulatory stimuli of receptor genes for pharmacologically important ligands would be of more than academic interest, if either such stimuli were used to induce the expression of drug receptors in order to achieve a better effect of the ligand, or if the effect of such stimuli could help to explain a drug’s side effects.

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References

22. Gunzer, M., Schafer, A., Borgmann, S., Grabbe, S., Zanker, K. S., Brocker, E. B.,


43. Hoehe, M. R., Köpke, K., Wendel, B., Rohde, K., Flachmeier, C., Kidd, K. K., Berrettini,
59. Li, H. L., Kostulas, N., Huang, Y. M., Xiao, B. G., van der Meide, P., Kostulas, V.,
Footnote
* Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte/macrophage colony-stimulating factor; HSV, Herpes simplex virus; IL, interleukin; nt, nucleotide; PMN, polymorphonuclear leukocytes; RT PCR, reverse transcription polymerase chain reaction; SEM standard error of the mean; Th, T helper cell; tk, thymidine kinase.

Figure Legends

Figure 1: Detection of IL-4 induced mu opioid receptor transcripts in immune effector cells
A: cDNAs from IL-4 stimulated (+) and unstimulated (-) immune cells (as indicated) and from SH SY5Y cells were subjected to RT PCR for the mu opioid receptor. The arrow indicates specific 342 bp amplification products. Lane M shows the φX174 DNA-HaeIII digest size marker. B: Detection of mu opioid receptor transcripts in undifferentiated monocytes (lane 1), cells during maturation (lanes 2 to 4) and differentiated dendritic cells (DC, lanes 5 and 6). Cells were incubated with GM-CSF (open bars) and/or IL-4 (black bars) according to the schedule under the gel followed by RT-PCR (corresponding gel lane above).

Figure 2: Real-time RT-PCR for mu opioid receptor mRNA isolated from primary cultured cortical neurons from rats after stimulation with IL-4.
The figure shows an example of a typical experiment. Cells were stimulated with IL-4 for different periods of time (as indicated) and then subjected to real time RT-PCR. The
experiments were performed in duplicate. A: Agarose gel of the real time PCR products. The arrow indicates 315 bp fragments of the rat mu opioid receptor. B: Diagram of the corresponding quantification results of the real-time PCR expressed as percent of unstimulated controls. Data are given as mean +/- SEM.

Figure 3: Inducibility of human and rat mu opioid receptor gene promoters by IL-4 in various cell lines.

CAT reporter gene activities for (A) human mu promoter construct phMOR-2229, (B) rat construct prMOR-1660 and (C) pBLCAT2 are shown as fold induction (white bars: unstimulated controls; grey bars: IL-4 stimulated transfectants). The results of at least three independent transfection experiments performed in triplicate plus SEM are plotted. For statistical evaluation see "Experimental Procedures" section.

Figure 4: Localization of IL-4 responsive promoter elements.

A: Transfection of human mu receptor constructs, depicted schematically relative to the promoter. The location of putative STAT6 binding sites (oval symbols) are indicated by vertical dashed lines. CAT activities for various promoter constructs, measured in Raji (R) and SH SY5Y (S) cells, are shown as fold induction (white bars: unstimulated controls; grey bars: IL-4 stimulated transfectants). Results of at least three independent experiments performed in triplicate plus SEM are plotted. For statistics see "Experimental Procedures" section. B: Transfection of rat mu receptor constructs. C: Transfection of constructs with IL-4 responsive elements in front of the HSV tk promoter. CAT activities are reported as fold inductions as above. The -997 element of the human (h) and the -727 element of the rat (r) mu receptor promoter were tested in sense (S) and antisense (A) orientation. Upper case letters of the sequences shown in the figure represent palindromic nucleotides. For the complete sequence of the oligonucleotides see "Experimental Procedures" section.

Figure 5: EMSAs demonstrating binding of STAT6 to the human (A) and rat (B) mu opioid receptor IL-4 responsive elements.
Lanes 1 contain labeled probe (indicated on the bottom of the gels) alone. In lanes 2 to 10, probes were incubated with IL-4 stimulated nuclear extract of SH SY5Y cells in the absence (-) or presence of competitor DNA as indicated above the gels. Two prominent complexes, C1 and C2, of which C1 is the STAT6 complex, are indicated by arrows. Unspecific competitor DNA (US) was used at 50-fold molar excess. Other competitors were added at 5- and 50-fold molar excess, as indicated by a triangle. Supershift experiments are depicted in lanes 11 (without) and 12 (with STAT6 antibody).

Figure 6: Effect of a polymorphism within the human -997 IL-4 responsive element.
A: Determination of the trans-activating potentials in response to IL-4 of the wild type (WT) and polymorph (PO) -997 elements of the human mu receptor promoter in sense (s) and antisense (a) orientation. See also legend to fig. 4. Statistical evaluation between these genetic variations (indicated by brackets) are plotted as "a" for Raji and "b" for SH SY5Y cells (p<0.001). B: Comparison of wild type and polymorph motifs with respect to their ability to compete for the classic STAT6 probe. Amounts of competitor DNA were: Lanes 1 and 6: no competitors, lanes 2 and 7: 2-fold molar excess, lanes 3 and 8: 4-fold molar excess, lanes 4 and 9: 8-fold molar excess, lanes 5 and 10: 16-fold molar excess. C: The genetic variant of the -997 motif (PO) is also a STAT6 binding site. See also legend to fig. 5.
Kraus et al. Figure 1

A

B

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Figure 3

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A

B

C

phMOR-2229

prMOR-1660

pLCAT2

Raji

U-937

IMR-32

SM/SY

HEK-293
Kraus et al. Figure 4

A

1. -2229
2. -1372
3. -779
4. -2229A-1854/-1372
5. -2229A-1933/-1033
6. -2229A-1372/-254
7. -1372A-1001/-950

B

1. -1660
2. -1198
3. -974
4. -605
5. -1660A-851/-445

C

1. h-997 S 5'-tCTTCtGAAGAg-3' th CAT
2. h-997 A 5'-gCTTCTgAGAAAGa-3'
3. r-727 S 5'-cCTTCcagaGAAAGa-3'
4. r-727 A 5'-tCTTCtctgGAAAGg-3'
Kraus et al. Figure 6

A

1. \(h_{-997} \text{WT-S} \quad 5'\text{-tCTTCTcAGAAAGc-3'}\) tk CAT
2. \(h_{-997} \text{WT-A} \quad 5'\text{-gCTTCTgAGAAGc-3'}\)
3. \(h_{-997} \text{PO-S} \quad 5'\text{-tCTTaTcAgAAAGc-3'}\)
4. \(h_{-997} \text{PO-A} \quad 5'\text{-gCTTcTgAAGAc-3'}\)

B

- Wild type -997
- Polymorph -997

C

- Classic probe
- -997 PO probe

class Ab Ab
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