KEPI, a PKC-dependent Protein Phosphatase 1 Inhibitor Regulated by Morphone*

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Agonists at many G protein-coupled receptors activate Gi and Go proteins, which in turn inhibit adenylate cyclase, alter fluxes through calcium and potassium channels, and enhance phospholipase activities that release activators of calcium/phospholipid-dependent protein kinase (PKC)1 (1–7). Second messenger changes caused by activation of Gi/Go-coupled receptors can also change levels of gene expression.

Morphine activation of Gi/Go-coupled receptors alters second messengers, ion fluxes, and gene expression patterns in ways that could contribute to long term consequences of µ receptor occupancy such as tolerance, dependence, and addiction (8–10). We have developed and used modifications of subtractive hybridization and differential display (SDD)-PCR approaches to seek morphine-regulated genes. These approaches thus provide a number of short cDNAs that correspond to morphine-regulated mRNAs.

To define interesting novel genes that might correspond to these apparently morphine-regulated cDNAs, we have screened mouse and rat brain cDNA libraries with probes derived from pools of short cDNAs that were morphine-regulated in initial SDD experiments. One of these cDNAs hybridized to a novel 2.6-kb mRNA species that was expressed specifically in brain, heart, and muscle and was up-regulated by acute and chronic morphine treatments in brain. The sequence of this mRNA displayed homologies with the previously elucidated CPI-17 and PHI/PNG genes (11, 12), which are PKC-potentiated inhibitors of the major serine/threonine type 1 protein phosphatase, PP1. We now report elucidation of this novel gene, termed “KEPI” (kinase-enhanced PP1 inhibitor), describe its ability to serve as a PKC substrate, and document its phosphorylation-dependent ability to inhibit PP1 selectively.

Definition of this protein and related sequences provides an expanded view of an emerging family of potential PKC-dependent PP1 inhibitors. Elucidating KEPI regulation by morphine also provides a novel mechanism for longer term effects of morphine on a variety of cellular signaling pathways, including those that might contribute to tolerance, dependence, and addiction.

EXPERIMENTAL PROCEDURES

Morphine Treatment, SDD, and RNA Analyses—Male Sprague-Dawley rats (250–300 g), wild type C57BL/6J mice, and µ receptor knockout mice (13) were housed under 12 h light/dark cycles. Rats were injected intraperitoneally with 20 mg/kg morphine or saline, sacrificed 4 h later, and their brains were removed rapidly. Frontal cortex, striatum, hippocampus, and thalamus were dissected on ice, and tissues were frozen in liquid nitrogen. For chronic morphine treatments, rats or

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1 The abbreviations used are: PKC, calcium/phospholipid-dependent protein kinase (protein kinase C); EST, expressed sequence tag; iKEPI, immunoreactive KEPI; KEPI, kinase-enhanced protein phosphatase type 1 inhibitor; PKA, cAMP-dependent protein kinase (protein kinase A); PP1, PP2, protein phosphatase types 1 and 2, respectively; SDD, subtractive hybridization and differential display; UTR, untranslated region.
mice were implanted subcutaneously with pellets containing 75 or 25 mg of morphine, respectively, or matched placebo controls (National Institute on Drug Abuse, Division of Basic Research). Animals were sacrificed 4 days after implantation, and brain regions (rat) or whole brains (mice) were dissected. RNA was isolated from frozen brain tissues by metal chelation chromatography as described (HisTrap kit, Amersham Biosciences). RNA was subjected to dideoxynucleotide dye termination sequencing using 373A automatic DNA sequencing and primer Walking (PE Applied Biosystems, Foster City, CA). Sequencer (Version 3.0, Gene Code Co.) assem- bled sequence data and GCG and NCBI tools provided comparisons. Automatic DNA sequencing and primer walking (PE Applied Biosys- tems, Foster City, CA). Sequencer (Version 3.0, Gene Code Co.) assem- bled sequence data and GCG and NCBI tools provided comparisons. We focused on a 2.2-kb murine cDNA insert that we termed initially B16.

Production and Purification of a Fusion Protein Including the B16 Open Reading Frame (His-KEPI)—A translation initiation site adaptor with sequence GGGCGCGGCGCATCGCGGTGTT, which provided a new NcoI restriction site (underlined), was used to amplify a 2-kb segment of the B16 cDNA clone including its entire open reading frame. PCR products were digested with Ncol at the translation initiation site and HindIII at the 3′-UTR end of the sequence and cloned into NcoI/HindIII sites of the bacterial expression vector pET30a (Novagen, Madison, WI) to form pET30aB16. The initiation methionine of pET30aB16 was positioned so that the B16 open reading frame followed the His tag peptide in-frame. BL21(DE3) Escherichia coli cells were transformed with pET30aB16, grown at 37°C in LB/kanamycin for 2–3 h, induced using 1 mM isopropyl-1-thio-β-D-galactopyranoside, grown at 37°C for 3 h, lysed by sonication, boiled for 5 min, and centrifuged at 10,000g for 10 min. Protein samples were subjected to dodecane sulfate gel electrophoresis (SDS-PAGE) (12) followed by Western blotting using rabbit antiserum diluted 1:1,000-fold for detection of iKEPI using alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody as described previously (15).

RESULTS

Identification of Mouse, Rat, and Human B16-KEPI cDNA Sequences and Initial Polymorphisms—cDNA clones that hybridized to pooled radiolabeled SDD probes were found in both rat and mouse brain cDNA libraries. Positive clones were further selected by hybridization with single short cDNAs. One of the longest inserts was the 2201-nucleotide clone B16. This sequence displayed a consensus sequence for translational ini- tiation (19). No in-frame stop codon was found in the 5′-regions of clone B16. Alignment of mouse EST sequences AL605782 and AA671272 provided an additional 390 nucleotides and a 5′-UTR to form an extended B16 cDNA. This extended B16 cDNA contains 2546 nucleotides and an in-frame stop codon located at nucleotide position 335 in the 5′-UTR of this se- quence. 164 amino acids are encoded between the translation initiation codon (ATG) at nucleotide position 518 and the trans- lation termination codon (TGA) at nucleotide position 1010 (Fig. 1). A poly(A) signal (AATAAA) is located at nucleotide positions 2507–2512, 13 bp upstream of the poly(A) tail (20). An additional poly(A) signal (AATAAC) is located at nucleotide position 1096–1101, 17 bp 5′ from the shorter poly(A) site at 1118. Mouse and rat EST sequences display both sites for poly(A) addition, potentially varying the lengths of their 3′-UTRs. Rat sequences were derived from a partial rat B16 cDNA whose 5′-sequence was augmented by information from

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Novel Member of the PKC-dependent PP1 Inhibitor Gene Family

Fig. 1. cDNA and deduced amino acid sequences for mKEPI. Nucleotide sequences are numbered in the left and right columns; amino acid sequences are numbered in brackets. The consensus Kozak sequence for translation initiation and poly(A) sequences are underlined. Potential PP1 docking motifs (RVFFQ) in the coding sequence and tetranucleotide repeat sequences (CCTT) in the 3′-untranslated region (3′-UTR) are in boldface. Carets indicate potentially N-myristoylated glycine residues. The asterisk (*) indicates the threonine residue potentially phosphorylated by PKC. The plus sign (+) indicates the serine residue potentially phosphorylated by casein kinase II. The numbers sign (#) indicates the serine residue potentially phosphorylated by PKA.

rat EST sequences BF567111 and BF560736. The extended rat cDNA sequences encode an open reading frame of 164 amino acids which displays 96% identity with mouse B16 amino acid sequences. The calculated molecular masses of the mouse and rat translation products are 17.75 and 17.98 kDa, respectively. The consensus Kozak sequence, no clear transmembrane domain, and no clear sites for N-linked glycosylation. The predicted protein is acidic, with an isoelectric point of 5.22 (Genetics Computer Group). The N-terminal residues 20–24, RVFFQ, contain a basic amino acid followed by two hydrophobic residues, recently described as a consensus PP1 binding motif (21). The N-terminal 50 amino acids could form a secondary structure with 12 predicted turns interrupting a more regularly spaced β-sheet structure (Genetics Computer Group). 10 N-terminal region glycine residues, Gly at positions 6, 7, 12, 13, 14, 15, 28, 31, 32, and 35, provide potential N-myristoylation sites (Genetics Computer Group Motif). There is a predicted 45% chance that C-terminal region amino acids 90–140 form a coiled-coil structure (Genetics Computer Group). Potential phosphoacceptor sites for PKC are identified at the Thr-72, for PKA at Ser-154, and for casein kinase II at Ser-43 and Ser-120 (Fig. 1). Each of these potential phosphorylation and acylation motifs is conserved in human, mouse, and rat B16 sequences (Fig. 2A).

Evolutionary Analysis of B16 as KEPI, a Member of the PKC-potentiated PP1 Inhibitor Protein Family—Murine B16 sequences from residues 70 to the C terminus are 67% identical to those of human messenger RNAs (mRNAs) (Fig. 2B). The 13 C-terminal amino acids near the Thr-72 potential site for PKC activity are absolutely conserved in each member of this gene family (Fig. 2B). KEPI mRNA Expression and Up-regulation—Northern analyses using a radiolabeled hybridization probe consisting of a 700-bp EcoRI fragment from the mouse B16-KEPI 3′-UTR just 3′ to the shorter polyadenylation signal site detect a 2.6-kb mRNA expressed in several brain regions, spinal cord, heart, and muscle (Fig. 3A). A 270-bp radiolabeled rat hybridization probe containing sequences 5′ to the shorter poly(A) signal site recognizes an additional, less intensely expressed 1.2-kb mRNA, consistent with the lower level expression of a shorter mRNA with alternative polyadenylation (data not shown). KEPI mRNA was detected in heart and muscle, but not in liver, kidney, testis, ovary, intestine, stomach, or spleen (some hybridization data not shown).
KEPI mRNA is up-regulated in mouse brain after chronic morphine treatment (Fig. 3B). KEPI mRNA is regulated differentially in different rat brain regions after acute and chronic morphine treatments (Fig. 3C). After acute morphine treatments, KEPI is most prominently up-regulated in hippocampus (120 ± 16%, n = 5). After chronic morphine treatment, KEPI is up-regulated most significantly in thalamus (75.6 ± 6.2%, n = 5). In brains of homozygous receptor knockout mice, however, expression is no different from expression in wild type mice (Fig. 3B).

**Association of Endogenous KEPI with Membranes**—Western analyses of iKEPI revealed bands with apparent mobilities of 45 and 37 kDa on SDS-PAGE bands. Immunoreactivities in both bands were blocked equally by preadsorption of the serum with the immunizing peptide. Immunoreactivities of both apparent mobilities were detected in rat cerebral cortex, midbrain, thalamus, brain stem, cerebellum, and heart. Faint 37 kDa bands were detected in proteins extracted from muscle, lung, liver, kidney, spleen, and testis (Fig. 4). No significant cross-reactivities with the 20 or 23 kDa SDS-PAGE mobilities of CPI and PHI, respectively (11, 26), were noted in these brain extracts.

KEPI immunoreactivity was associated with membrane fractions and little with cytosolic fractions. The higher molecular mass 45-kDa immunoreactive species was much more prominent in the P2 crude synaptosomal fraction, whereas lower molecular mass 37-kDa species was found in the P3 crude microsomal fractions (Fig. 4). However, after prolonged storage in SDS sample buffer, much of the iKEPI in extracts from P2 fraction migrated at the 37 kDa position of the lower band (data not shown). Molecular masses observed in Western analyses could be higher than those calculated from KEPI peptide sequence lengths because of deviant SDS-PAGE migration of acidic polypeptides (27) and/or post-translational modifications of KEPI. Differential migration displayed by iKEPI extracted from different subcellular fractions supports substantial roles for post-translational modifications.

Preparation and Purification of mKEPI Fusion Protein—A recombinant murine KEPI fusion protein consisting of KEPI fused to 43 pET30a vector amino acids including its His tag,
thrombin recognition sequences, and enterokinase recognition sequences yielded a 32 kDa band after SDS-PAGE of extracts of isopropyl-1-thio-β-D-galactopyranoside-induced expressing BL21 E. coli (Fig. 5, left). This was again larger than the 23-kDa unmodified mass predicted from the sequence as often found for acidic proteins. The heat stability of KEPI allowed cell extract boiling and HisTrap metal chelation chromatography, facilitating purification of this fusion protein to homogeneity (Fig. 5, right). Yields of His-KEPI reached as high as 10 mg of protein/liter of bacterial culture.

**PKC Phosphorylation of KEPI**—Recombinant His-KEPI protein can be phosphorylated by purified rat brain PKC preparations. Under these conditions, recombinant protein was phosphorylated with apparent $K_m = 2.6 \mu M$, $V_{max} = 37.8$ nmol/mg/min, and $t_{1/2}$ of 20.7 min (Fig. 6A). At plateau time points, a near stoichiometric ratio of phosphorylation, $0.81 \pm 0.12$ mol of phosphorus/mol of His-KEPI was achieved. When the mobility of $^{32}$P-labeled His-KEPI was analyzed by SDS-PAGE and autoradiography (Fig. 6B), a dominant 32 kDa $^{32}$P-labeled band corresponding to His-KEPI was observed.

**Inhibition of PP1 Dephosphorylation Activity by KEPI**—Phospho-KEPI, produced by KEPI preincubation with PKC, inhibited the ability of rabbit muscle PP1α to dephosphorylate $^{32}$P-labeled phosphorylase. This activity was inhibited by PKC-phosphorylated recombinant KEPI fusion protein with an IC$_{50}$ of $2.7 \pm 0.7$ nM (Fig. 7A). Unphosphorylated KEPI could only inhibit rabbit muscle PP1α at much higher concentrations, displaying IC$_{50}$ values of 1.8 $\mu$M. KEPI thus increased its in-
hitory potency for PP1α more than 600-fold after phosphorylation by PKC. However, neither the phosphorylated nor unphosphorylated KEPI form was able to inhibit bovine kidney PP2A1-mediated dephosphorylation of the same 32P-labeled phosphorylase substrate (Fig. 7B). Indeed, high nanomolar concentrations of phospho-KEPI slightly enhanced PP2A1 activities.

**KEPI Genomic Structure**—The three exons encoding the human KEPI C-terminal polypeptide sequences are found among GenBank sequences AL096708.34 of BAC RP5-1179L24 and Celera hCG166079, localized to chromosome 6q24.3–25.3. The first exon encoding the KEPI N-terminal peptide sequence is found in GenBank sequences AL138890.9 and AL355497 of the overlapping BACs RP5-932N18 and RP11-472G23, as well as Celera genomic scaffold Gax54KREAVE16.1 which again localized to 6q25.2–26. The genomic contig AL096708 contains each of these BAC sequences, sequence-tagged sites including stSG9583, W1-22584, stSG27036, stSG1514, stSG6339, and stSG9891 and links to ESTs of the UniGene Hs.12599 locus. We assembled sequences of the three overlapping genomic BAC clones covering approximately 330 kb and identified four KEPI exons on more than 100 kb of chromosome 6 sequence (Fig. 8). The sequences of predicted exon/intron junctions are shown in Table II. Intron 1 is 71 kb, intron 2 is 2 kb, and intron 3 is 32 kb in length. Neighboring genes can also be tentatively identified. Exon sequences that display homologies to the major histocompatibility complex class I-related ligand UL16-binding protein gene, hypothetical genes LOC135250, LOC135251, LOC135252, LOC135254, LOC135256, and processed pseudogenes for RNA polymerase B transcription factor 3 (BTF3) and prohibit (PHB) were identified 5′ to the KEPI gene. The hypothetical gene LOC135258 was identified 3′ to KEPI gene in these BAC sequences. Interestingly, KEPI maps on chromosome 6 at 317.7 megabases, 2 megabases centromeric to the 161.5-megabase locus for the μ receptor OPRM1 (AL136444) locus and is transcribed in the same orientation, toward the 6q telomere (NCBI human genome map viewer build 27).

**DISCUSSION**

Drug-regulated genes include several whose products are involved in signals emanating from activated G/Go-coupled receptors. These include the G protein β1 subunit (14), PP2B/calcineurin (8), and the novel kinase-enhanced PP1 inhibitor KEPI described here. Elucidating these genes raises interest in roles that they or their family members might play in normal cellular functions and in drug-altered functions including those that contribute to addictions.

One approach to these pathways examines the balance between protein kinase and phosphatase activities which regulates dynamic protein phosphorylation process (28). PP1 and PP2A are among the most prominent Ser/Thr protein phosphatases in mammalian cells and can be distinguished from types 2B and 2C phosphatase by their preferential dephosphorylation of phosphorylase A (17). PP1 is thus a signal-transducing enzyme that can modulate the physiological activities of numerous proteins by regulating their states of phosphorylation or dephosphorylation. PP1 is enriched in neuronal postynaptic densities (29) and acts in neurons to regulate phosphorylation of many neurotransmitter receptors, voltage-gated ion channels, ion pumps, and transcription factors (30, 31). PP1 can also regulate other cellular processes, including protein synthesis, metabolism, muscle contraction, and cell division (17). The dephosphorylating activities of PP1 are regulated by a group of heat-stable inhibitory proteins (32). These PP1 inhibitory proteins are themselves phosphorylated and regulated by several protein kinases. PKA phosphorylates DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M7, 32,000), inhibitor-1, and NIPP-1 (nuclear inhibitor of PP1) (33). cGMP-dependent protein kinase (protein kinase G) phosphorylates G substrate (34). Glycogen synthase kinase-3 inhibitory proteins inhibit-2 (35). Calcium/phospholipid-dependent protein kinase (PKC) phosphorylates CPI-17, PNG/PHI, and KEPI (11, 36). PP1 inhibitory activities can be enhanced up to 1,000-fold upon phosphorylation of specific threonine residues in DARPP-32, inhibitor-1, G substrate, CPI-17, PHI/PNG and KEPI (11, 17, 26, 33). In contrast, phosphorylation of NIPP-1 and inhibitor-2 reduces their potencies in inhibiting PP1 (35, 37). The activities of PP1 inhibitory proteins are returned to the basal level by dephosphorylation (38) by several different phosphatases. Phosphatase 2B/calcineurin dephosphorylates DARPP-32 at its PKA phosphorylation site and virtually eliminates its PP1 inhibitory activity (39). PP1 inhibitory proteins could thus play intermediate regulatory roles between kinases and phosphatases to modulate phosphorylation states of multiple target molecules in response to extracellular signals. Observations that KEPI displays potential phosphorylation sites for PKA and casein kinase II could also indicate that KEPI may be under even more complex regulation by phosphorylation or dephosphorylation at several of its sites.

KEPI appears well positioned to play a significant role in inhibiting one of the major cellular phosphatases, PP1, when PKC is activated in brain or heart. Such activities are also displayed by several other members of its gene family. CPI-17 is expressed heavily in smooth muscle, where its activation by PKC phosphorylation makes it a potent inhibitor of PP1-myosin light chain complex. When phosphorylated by PKC, PNG/PHI inhibits myosin- and glycogen-associated PP1 holoenzyme activities in multiple tissues (11, 26). We have demonstrated here that recombinant KEPI fusion protein can specifically inhibit rabbit muscle PP1α after PKC phosphorylation. In this in vitro test, KEPI inhibition of phosphatase was just as potent as values reported for porcine CPI or human PHI (11, 12). In very recent initial work, we have also found that the KEPI family member FLJ29251/GCPII is also a potent PKC-dependent PP1 inhibitor found in tissues including gastrointestinal and cerebral cortex. Different tissue and subcellular localizations of members of this family of PKC-dependent PP1 inhibitors provide the opportunity to exert exquisite tissue-specific control over temporal profiles of PP1 activity.
Some of the detailed molecular mechanisms for KEPI inhibition of PP1 could share similarities with those of other family members. KEPI shares limited amino acid sequence identity with DARPP-32. By analogy to DARPP-32, a potential KEPI inhibitory domain surrounding a potential phospho-Thr-72 site might bind to the PP1 active site and inhibit its dephosphorylating enzymatic activity (40, 41). The N-terminal residues 20–24 (RVFFQ) of KEPI contain a consensus motif (basic amino acid followed by two hydrophobic residues) that could represent its PP1 binding domain (21). Conceivably, such a KEPI domain could allow it to compete for PP1 binding with its other partners, such as spinophilin, to alter the way in which PP1 could otherwise selectively dephosphorylate molecules such as ligand- and voltage-gated ion channels that are often located near spinophilin (42). In addition to this possible PP1 binding motif, the acidic cluster of KEPI residues 88–125 shares 50% similarity with DARPP-32 residues 104–141. Conceivably, the complex structure predicted for KEPI’s N-terminal 50 amino acids could allow its centrally localized PKC recognition site to be more accessible for phosphorylation and dephosphorylation. If KEPI were indeed membrane-anchored by myristoylation at its N-terminal glycine (43), its flexible N-terminal region might even allow the C-terminal coiled-coil structure predicted for KEPI to interact with other proteins.

The physiological in vivo significance of KEPI’s in vitro potencies could depend on several features, including the cellular levels of KEPI expression, repertoires of expressed PP1 isoforms (44), and expression of other members of this gene family. It will be interesting to compare its expression with those of the FLJ29251/GCPI protein and other members of the more acidic or basic subfamilies of kinase-enhanced PP1 inhibitors. PKC is a heterogeneous enzyme with a dozen isoforms implicated in many cellular responses (45). The exact repertoire of PKC isozymes expressed by the cells of interest could also influence in vivo activities of KEPI. PKC and KEPI subcellular localizations could also vary. Agonist activation of SH-SY5Y cell μ receptors can lead to membrane translocation and activation of the PKC calcium- and diacylglycerol-regulated form, its DAG-regulated ε form, and its ζ form (5, 46, 47). Subcellular localization could also be altered in vivo by differential N-terminal KEPI myristoylation. Acylation could change KEPI’s membrane anchoring and thus change its proximity to membrane-bound PKC. Differential phosphorylation by casein kinase II at the SXXE motifs found at Ser-43 and Ser-120 or by PKA at its RI/RRKS motifs, including Ser-154, could also contribute to in vivo variations in KEPI activities and enrich the current picture. Even information available to date indicates that the presence of KEPI could result in significantly lengthened half-lives of important protein phosphorylation events when PKC is activated.

Longer protein phosphorylation half-lives could be especially effective in amplifying and prolonging signals from agonist activation of Gq/Gi-coupled receptors when phosphorylation rates were also enhanced. Agonist-exposed cells that express Gq/Gi-coupled receptors and high levels of KEPI might thus accumulate many more phosphates on phosphoacceptor sites of...
important cellular proteins than agonist-exposed cells that contain the same receptors but lack KEPI or its family members. Such mechanisms could enhance “feed forward” mechanisms that could profoundly alter the dynamic properties of phosphorylation events.

These events could change still further after drug treatments. After opiate treatments, for example, cells with up-regulated KEPI expression could display more prominent effects. After opiate treatments, for example, cells with up-regulated KEPI expression could display more prominent effects. This up-regulated KEPI could increase the level of phosphorylation of neurotransmitters such as glutamate receptors, Ca2+ channels, GABA receptors, Na+ channels, Na+ /K+-ATPase (31), and other proteins. Phosphorylated signaling molecules are in turn candidates to contribute to behavioral manifestations such as sensitization, the greater locomotor stimulation that a repeated dose of morphine ut to behavioral manifestations such as sensitization, the greater locomotor stimulation that a repeated dose of morphine

regulating G protein-coupled receptors. Because these G/Gs-coupled receptors include many of the receptors that are activated directly or indirectly by most drugs of abuse, including opiates, cannabinoids, and psychostimulants, KEPI and its family members seem especially well positioned to play significant roles in these processes.

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Table II

| Exon | Exon size | Acceptor sequence | Exon sequence | Donor sequence | Intron size | Intron |
|------|-----------|-------------------|---------------|----------------|-------------|--------|
| 1    | >400      | tcccactgccccccag  | GAGAAGAGAAAAATTACA | gtaacgattccccaaat  | 71,251      | 1      |
| 2    | 84        | ttcttgtgttttaacctag | GAAGCTCTT—ACCAACAGA | gtaagaaatctttttcttc  | 1,995       | 2      |
| 3    | 33        | etcttgcagcttctttag | GAATTTATCA—CTGCTTACAA | gcctgttgacatgtgtggc  | 31,888      | 3      |
| 4    | 1,613     | etcttgcagcttctttag | GAATTTATCA—CTGCTTACAA | gcctgttgacatgtgtggc  | 31,888      | 3      |

![Image](242x648 to 554x729)

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