Enkephalins Are Transported by a Novel Eukaryotic Peptide Uptake System*

Melinda Hauser‡, Amy M. Donhardt‡, David Barnes‡, Fred Naider§, and Jeffrey M. Becker‡¶

From the §Department of Microbiology and Biochemistry, Molecular and Cellular Biology, University of Tennessee, Knoxville, Tennessee 37996-0845 and the ¶Department of Chemistry, College of Staten Island, City University of New York, Staten Island, New York 10314

We have identified an oligopeptide transporter in the yeast Saccharomyces cerevisiae which mediates the uptake of tetra- and pentapeptides, including the endogenous opioids leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) and methionine enkephalin (Tyr-Gly-Gly-Phε-Met). The transporter is encoded by the gene OPT1. Yeast expressing OPT1 can utilize enkephalins to satisfy amino acid auxotrophic requirements for growth. The transport of radiolabeled leucine enkephalin exhibits saturable kinetics, with a \(K_m\) of 310 \(\mu M\). Transport activity is optimum at acidic pH and sensitive to reagents which uncouple oxidative phosphorylation, suggesting an energy dependence on the proton gradient. Growth, transport, and chromatographic data indicate that leucine enkephalin is not hydrolyzed in the extracellular medium and as such is translocated intact across the cell membrane. The system is specific for tetra- and pentapeptides and can be inhibited by the opioid receptor antagonists naltroxone and naltrexone. To date, this is the first example of a eukaryotic transport system which can use enkephalins as a substrate, opening the possibility that a homologue exists in higher eukaryotes.

Small peptides containing four to five amino acid residues are transported by a recently identified class of peptide transporters named the OPT family (1, 2). The amino acid sequence of this family is distinct from that of the PTR family, a ubiquitous group of proton-coupled transporters which selectively transports di- and tripeptides (3, 4). Phylogenetic analysis suggests that the OPT family is also distinct from the major facilitator superfamily (MFS), a diverse collection of proteins which catalyzes the transport of a wide variety of substrates, including sugars, amino acids, neurotransmitters, and drugs (5).

Members of the OPT family have been identified and characterized in the yeasts Candida albicans, Schizosaccharomyces pombe, and Saccharomyces cerevisiae. Additional members exist in plants, as indicated by searches of publicly accessible data bases. In mammalian tissues, reports in the literature suggest that the enkephalins, endogenous pentapeptides involved in analgesia in the central nervous system, are transported across the blood-brain barrier by a specific, saturable transport system (6). The existence of enkephalin transporters has been inferred from data obtained by measuring whole brain flux of the peptides in rodents (7–10). To date, no protein has been identified in eukaryotes as the discrete enkephalin carrier.

In this paper, we report that the endogenous opioids Met-enkephalin and Leu-enkephalin, pentapeptides of amino acid sequence YGGFM and YGGFL, respectively, can be transported by cells expressing the S. cerevisiae ORF YJL212C. When expressed under the control of a constitutive promoter in a high copy number vector, this OPT family member is necessary and sufficient to transport Leu-enkephalin into yeast cells. This is the first example of a genetically defined eukaryotic transport protein which can transport enkephalins across the cell membrane. In accordance with the standard nomenclature for S. cerevisiae, we propose the name OPT1 for this gene.

EXPERIMENTAL PROCEDURES

Strains, Media, and Vectors—The strains used in this study were obtained from Dr. Phillip Heiter (11). BY4700 (Mata leu2Δ0 met15Δ0 ura3Δ0) and BY4730 (Mata leu2Δ0 met15Δ0 ura3Δ0) were grown routinely on YEPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar). The strains used in this study were

### EXPERIMENTAL PROCEDURES

*This work was supported by Grants GM22086 and GM22087 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Dept. of Microbiology, M409 Walters Life Sciences, The University of Tennessee, Knoxville, TN 37996. Tel.: 865-974-3006; Fax: 865-974-4007; E-mail: jbecker@utk.edu.

‡From the Department of Microbiology and Biochemistry, Molecular and Cellular Biology, University of Tennessee, Knoxville, Tennessee 37996-0845 and the Department of Chemistry, College of Staten Island, City University of New York, Staten Island, New York 10314.

§To whom correspondence should be addressed: Dept. of Microbiology, M409 Walters Life Sciences, The University of Tennessee, Knoxville, TN 37996. Tel.: 865-974-3006; Fax: 865-974-4007; E-mail: jbecker@utk.edu.

¶The abbreviations used are: OPT, oligopeptide transporter; Leu-enkephalin, leucine enkephalin; Met-enkephalin, methionine enkephalin; CCF, carbonyl cyanide 3-chlorophenylhydrazone; pCMBS, 4-chloromercuribenzenesulfonic acid; DADLE, Tyr-D-Ala-Gly-Phe-D-Leu; DP-DPE, Tyr-D-Penicillamine-Gly-Phe-D-Penicillamine; Tyr-MIF-1, tyrosine melanocyte-stimulating hormone inhibitory factor 1; ORF, open reading frame.
cells), and incubating at 30 °C. For determination of leucyl-leucine accumulation, 320 μM L-leucyl-l-[3H]leucine (16 nm, 10 mCi/mmol) was used in place of Leu-enkephalin. L-Leucyl-L-[3H]leucine was synthesized by standard solution-phase techniques (14). For assays done in the presence of competitors, the 2× uptake assay mixture was supplemented with competitor (2× final concentration) prior to combining with the cells. A concentrated stock of CCCP (Sigma) was prepared in Me2SO; naloxone and naltrexone (Sigma) were dissolved in methanol. The compounds were diluted such that the solvent was present at a final concentration of 5% in the uptake medium. All other compounds were dissolved in either water or sodium citrate/potassium phosphate buffer (pH 5.5). At the appropriate time, aliquots (90 μl) were removed and washed by vacuum filtration with 4 × 1 ml ice cold water onto a membrane filter (HAWP, Millipore). The membranes were counted by liquid scintillation spectrometry, and results were reported as nmol/mg dry weight. Data points reflect the mean and standard deviation of a minimum of four independent measurements.

Chromatography—Cells were incubated with uptake medium for 12 min, harvested, and washed four times with ice-cold water. The cell pellet was extracted by boiling in 50% methanol. The methanol extracts, along with control samples, were spotted onto silica plates and developed by ascending chromatography using butanol/glacial acetic acid:water solvent system (9:1:2.5). The chromatograms were sprayed with ninhydrin (0.1% in 95% ethanol) to visualize the nonradioactive acid:water solvent system (9:1:2.5). The chromatograms were sprayed with ninhydrin (0.1% in 95% ethanol) to visualize the nonradioactive

**RESULTS**

**Growth on Leu-Enkephalin**—An experiment was designed to determine whether members of the OPT family could transport leucine enkephalin (Leu-enkephalin; YGGFL) to satisfy an auxotrophic requirement for leucine. For this study, a strain of *S. cerevisiae* auxotrophic for methionine and leucine (BY4730) along with the prototrophic parental strain (BY4700) were selected for use (11). *S. cerevisiae* BY4730 transformed with the vector pDB20 and transformants expressing three members of the OPT family were able to use either leucine or leucyl-leucine for growth (Fig. 1). In contrast, only cells transformed with the vector pDB20 did not accumulate enkephalin. The uptake of Leu-enkephalin was pH-dependent. Transport of the substrate was highest at pH 5.5 and declined sharply as the proton concentration was raised or lowered (Fig. 2B). This pH optimum is similar to those reported for the eukaryotic di- and tripeptide transport systems (4, 15), as well as that for peptide transport in the prokaryote *Lactococcus lactis* (16). Treatment of cells with the metabolic uncouplers 2,4-dinitrophenol, CCCP, or sodium azide, all of which deplete intracellular ATP and collapse the proton gradient, or treatment with the sulfhydryl reagent pCMBS substantially reduced enkephalin uptake (Table I). These data are consistent with a carrier-mediated uptake system for Leu-enkephalin encoded by OPT1.

The rate of Leu-enkephalin uptake remained relatively constant over a 12-min time course, suggesting that the opioid does not remain intact upon entering the cell. Chromatographic analysis of radiolabeled material extracted from cells indicated that the enkephalin was degraded, with virtually all radioactivity associated with free tyrosine (Fig. 3). In contrast, analysis of an aliquot of medium from which cells were removed after 12 min of incubation at 30 °C revealed that no extracellular hydrolysis of the peptide had occurred. All radioactivity was still associated with intact Leu-enkephalin. If it is assumed that translocation of the substrate, rather than its
hydrolysis, is rate-limiting, then an apparent $K_m$ for transport can be determined by measuring the rate of transport as a function of substrate concentration. Transformation of these data gives an apparent $K_m$ of 310 μM for the uptake of Leu-enkephalin by transporter (Fig. 2A, inset).

The transport protein encoded by OPT1 has a strong preference for both Leu-enkephalin and Met-enkephalin and does not appear to transport amino acids or di- or tripeptides (Table I). Accumulation of Leu-enkephalin was not affected by the presence of tyrosine or the di- and tripeptides tested, suggesting that the OPT1 protein does not recognize these compounds. The uptake of radiolabeled Leu-enkephalin decreased by 75–88% in the presence of a 10-fold molar excess of Met-enkephalin or Leu-enkephalin, respectively. The tetrapeptide Lys-Leu-Gly-Leu (KLGL), a known substrate for other oligopeptide transporters (1, 2) was also an effective competitor. The amidated tetrapeptide Tyr-MIF-1 (YPLG-NH₂) showed weak inhibition of enkephalin uptake in yeast.

**TABLE I** Enkephalin Transport in Yeast

| Compound                          | Percent of control |
|-----------------------------------|--------------------|
| None                              | 100%               |
| Leucine Enkephalin (YGGLFL)       | 12 ± 1%            |
| Methionine Enkephalin (YGGFM)     | 25 ± 4%            |
| Tyrosine                          | 95 ± 12%           |
| Leu-Leu                           | 97 ± 12%           |
| Gly-Gly-Phe-Leu                   | 99 ± 5%            |
| Gly-Gly-Phe-Leu-Leu               | 41 ± 8%            |
| Lys-Leu-Gly-Leu                   | 31 ± 14%           |
| MIF-1 (PLG-NH₂)                   | 95 ± 7%            |
| Tyr-MIF-1 (YPLG-NH₂)              | 78 ± 9%            |
| Tyr-Gly-Gly-Phe-Leu-NH₂           | 71 ± 5%            |
| DPDPE (YD-Pen-GF-D-Pen)           | 69 ± 11%           |
| DADLE (YD-AGF-D-L)                | 58 ± 5%            |
| Sodium Azide                      | 21 ± 2%            |
| 2,4-Dinitrophenol                 | 17 ± 2%            |
| CCCP                              | 38 ± 6%            |
| pCMBS                             | 55 ± 5%            |

a All competitors were at a final concentration of 2.5 mM and added simultaneously with [3H]Leu-enkephalin in the uptake medium.

b Cells were pre-incubated with sodium azide (1 mM), CCCP (0.1 mM) 2,4-dinitrophenol (1 mM), or pCMBS (0.2 mM) for 30 min prior to addition of the uptake medium.

**Inhibition of Uptake by Enkephalin Analogs**—The nonmetabolized pentapeptide enkephalin analogues DADLE and DPDPDE were somewhat effective competitors, blocking 30–40% of the uptake (Table I). The amidated tetrapeptide Tyr-Leu-Gly-Leu (KLGL), a known substrate for other oligopeptide transporters (1, 2) was also an effective competitor. The amidated form of Leu-enkephalin (Tyr-Gly-Phe-Leu-NH₂) showed weak inhibition of enkephalin uptake in yeast.

**DISCUSSION**

In this paper we assign a function to the previously unknown open reading frame YJL212C in the yeast *S. cerevisiae* and have named this gene OPT1. The protein encoded by OPT1 consists of 799 amino acids, and based on the amino acid
sequence the predicted protein structure suggests an integral membrane protein containing 12–14 putative membrane-spanning domains. In addition, the protein contains several motifs unique to the OPT family, the largest of which consists of 10 invariant residues (SPYXEVXVXXXXDDP) located before the first hydrophobic domain (2). In this study we have confirmed that OPT1, like other members of the OPT family, encodes a functional oligopeptide transporter.

Because Opt1p exhibited all the molecular characteristics of an OPT family member, it was hypothesized that this protein was an oligopeptide transporter, even though it was known that S. cerevisiae could not utilize any tetra- or pentapeptides tested to date to satisfy auxotrophic requirements under routine growth conditions (1, 19). To see activity of Opt1, it was necessary to express OPT1 under the control of the ADH promoter, a strong, constitutive promoter which would presumably result in high expression of the gene product. In prior studies, Northern blot analysis confirmed that OPT1 was not expressed at detectable levels under routine conditions of logarithmic growth (2). These results were independently confirmed by serial analysis of gene expression (SAGE) (20) which revealed that OPT1 is only expressed at a low level (~1 copy per cell) following nocodazole arrest in the G 2/M phase of the cell cycle. Additional analysis of sporulating yeast cells by DNA microarray analysis indicated that OPT1 was expressed during the late stages of sporulation (21). In light of these observations, OPT1 gene expression had to be ectopically induced under the control of a heterologous promoter to enable study of Opt1p function in log phase cells.

The product of OPT1 is the oligopeptide transporter Opt1p, which translocates pentapeptides, including both Met- and Leu-enkephalin. In BY4730, a strain of S. cerevisiae auxotrophic for leucine and methionine, only cells expressing OPT1 could grow on Leu-enkephalin in the absence of exogenous leucine. This indicates that enkephalins are transported intact into the cell and then hydrolyzed. If oligopeptides were hydrolyzed by an extracellular protease prior to transport, then the isogenic control strain (BY4730 transformed with the empty vector pDB20), as well as yeast cells transformed with plasmids encoding other OPT family members (CaOPT1, YPR194C) should be able to utilize the hydrolysis products for growth. Chromatographic analysis supports this postulate; no evidence for degraded forms of Leu-enkephalin could be found in the extracellular medium. In addition, a large body of work exists which demonstrates that di- and tripeptides enter the cell intact and are then rapidly hydrolyzed by intracellular peptidases (19).

Transport of Leu-enkephalin is pH- and temperature-dependent, suggesting that this is a proton-coupled, energy-dependent process. These observations are supported by the sensitivity of the transporter to agents which disrupt the proton gradient or deplete intracellular ATP. Utilization of the transmembrane proton gradient to energize active transport has been demonstrated for the PTR family of di- and tripeptide transporters (4). Uptake of radiolabeled Leu-enkephalin was inhibited in the presence of excess unlabeled Met- or Leu-enkephalin; amidated Leu-enkephalin was an ineffective competitor, Tyr-MIF-1 is an amidated tetrapeptide with opiate and anti-opiate activity. This peptide is a substrate for the previously described blood-brain barrier PTS-1 enkephalin transport activity (6) but, like the amidated form of authentic Leu-enkephalin, was not an effective competitor for yeast Opt1p. This observation is consistent with the need for a free carboxyl terminus for substrate recognition by Opt1p. Tetrapeptides were effective inhibitors, with Lys-Leu-Gly-Leu and des-Tyr1 Leu-enkephalin (Gly-Gly-Phe-Leu) eliminating over 50% of radiolabeled enkephalin accumulation, suggesting that an amino-terminal tyrosine is not essential for substrate recognition. Neither the tripeptide enkephalin fragment Gly-Gly-Phe nor the dipeptide Leu-Leu could inhibit uptake, indicating that this system is distinct from Ptr2p and is selective for tetra- and pentapeptides. These data suggest that intact oligopeptides are gaining access to the cell via a carrier-mediated process and that the discrete carrier is the gene product of OPT1. If enkephalins were entering by a nonspecific mechanism such as simple diffusion or endocytosis, then all strains, not just those expressing OPT1, should be able to utilize this substrate.

Several enkephalin antagonists were assayed in this study for their effect on enkephalin transport across Opt1p. DADLE and DPDPE are enzymatically stable delta opioid receptor antagonists that are pentapeptide mimetics. Previous reports indicated that DPDPE gained access to the brain by a saturable, carrier-mediated mechanism in the blood-brain barrier, which has yet to be defined (22, 23). Interestingly, transport of DPDPE was not inhibited by Leu-enkephalin in those studies, suggesting either the existence of separate transport systems or a common system with different affinities for these two substrates. A recent report suggests that DPDPE crosses the blood-brain barrier by a phenylarsine oxide-sensitive pathway, suggesting a role for a saturable endocytic mechanism in the in vitro and in situ models studied (24). In the present study, DPDPE and DADLE were weak competitors for Leu-enkephalin transport, indicating that Opt1p interacts with the stable antagonists with differential affinities compared with authentic Leu-enkephalin.

Naloxone and naltrexone are synthetic opioid receptor antagonists classically used to reverse the effects of opiate overdose (18). Naltrexone is also used clinically in the treatment of alcoholism. Despite the fact that these compounds are similar in structure to morphine, rather than resembling a peptide, they were effective competitors for Leu-enkephalin transport. The effect appears to be specific for the Opt1p transporter because the presence of the morphine analogs did not influence the activity of the unrelated di- and tripeptide transporter Ptr2p. The nature of the inhibition of Leu-enkephalin transport by naloxone and naltrexone is currently under investigation. Specifically, it would be of interest to determine whether these compounds are substrates for transport or are nonsubstrate competitors for Opt1p.

There is increasing evidence that opioids and their analogues enter the central nervous system by carrier-mediated transport across the blood-brain barrier (6, 22, 25). Evidence also exists to suggest that the clearance of the enkephalin analogue DPDPE occurs by saturable eflux from the brain and systemic elimination of intact DPDPE via biliary excretion (26). Furthermore, it is possible that neuronal re-uptake systems exist for enkephalin similar to the well studied transport systems for neurotransmitters such as serotonin and γ-aminobutyric acid (27, 28). To date, none of the putative transporters for enkephalin have been cloned or characterized at a molecular level. In this report, we present the first evidence for a genetically defined eukaryotic transport protein, Opt1p, which recognizes and translocates both Met- and Leu-enkephalin into an intact eukaryotic cell. The identification of this transporter in Saccharomyces may facilitate the discovery of mammalian homologues, thus providing greater insight into the process of pain and its mediation.

Acknowledgment—We thank Michael Owston for assistance.

REFERENCES
1. Lubkowitz, M. A., Hauser, L., Breslav, M., Naider, F., and Becker, J. M. (1997) Microbiology 143, 387–396
2. Lubkowitz, M. A., Barnes, D., Breslav, M., Burchfield, A., Naider, F., and
Enkephalin Transport in Yeast

Becker, J. M. (1998) Mol. Microbiol. 28, 729–741
3. Steiner, H.-Y., Naider, F., and Becker, J. M. (1995) Mol. Microbiol. 16, 825–834
4. Fei, Y. J., Ganapathy, V., and Leibach, F. H. (1998) Prog. Nucleic Acids Res. Mol. Biol. 58, 239–261
5. Steiner, H.-Y., Naider, F., and Becker, J. M. (1995) Mol. Microbiol. 16, 825–834
6. Fei, Y. J., Ganapathy, V., and Leibach, F. H. (1998) Prog. Nucleic Acids Res. Mol. Biol. 58, 239–261
7. Banks, W. A., and Kastin, A. J. (1997) Alcohol 14, 237–245
8. Plotkin, S. R., Banks, W. A., Waguespack, P. J., and Kastin, A. J (1997) J. Neurosci. Res. 48, 273–280
9. Slavkovic, B. V., Mackie, J. B., Djuricic, B. M., and Davson, H. (1989) J. Neurochem. 53, 1333–1340
10. Egleton, R. D., Abruscato, T. J., Thomas, S. A., and Davis, T. P. (1996) J. Pharm. Sci. 87, 1433–1439
11. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 115–132
12. Becker, D. M., Fikes, J. D., and Guarente, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 5, 1991–1995
13. Gietz, R. D., and Schiestl, R. H. (1995) Meth. Mol. Cell Biol. 5, 255–269
14. Becker, J. M., and Naider, F. (1997) Arch. Biochem. Biophys. 178, 245–255
15. Roman, G., Meller, V., Wu, K. H., and Davis, R. L. (1999) Am. J. Physiol. 275, C857–C869
16. Hagting, A., Kunji, E. R. S., Leenhouts, K. J., Poolman, B., and Konings, W. N. (1994) J. Biol. Chem. 269, 11391–11399
17. Banks, W. A., Kastin, A. J., Fischman, A. J., Coy, D. H., and Strauss, S. L. (1986) Am. J. Physiol. 251, E477–E482
18. McNicholas, L. F., and Martin, W. R. (1984) Drugs 27, 81–93
19. Becker, J. M., and Naider, F. (1995) in Peptide-based Drug Design: Controlling Transport and Metabolism (Taylor, M. D., and Amidon, G. L., eds) pp. 369–384, American Chemical Society, Washington D. C.
20. Veleloucas, V. E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M. A., Basset, D. E., Jr., Hieter, P., Vogelstein, B., and Kinzler, K. W. (1997) Cell 88, 243–251
21. Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O., and Herskowitz, I. (1998) Science 282, 699–705
22. Thomas, S. A., Abruscato, T. J., Hruby, V. J., and Davis, T. P. (1996) J. Pharmacol. Exp. Ther. 280, 1235–1240
23. Williams, S. A., Abruscato, T. J., Hruby, V. J., and Davis, T. P. (1996) J. Neurochem. 66, 1289–1299
24. Egleton, R. D., and Davis, T. P. (1999) J. Pharm. Sci. 88, 392–397
25. Fiori, A., Cardelli, P., Negri, L., Savi, M. R., Strom, R., and Erspamer, V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9469–9474
26. Chen, C., and Pollack, G. M. (1997) J. Pharmacol. Exp. Ther. 283, 1151–1159
27. Blakely, R. D., Ramamoorthy, S., Schroeter, S., Qian, Y., Apparsundaram, S., Galli, A., and DeFelice, L. J. (1998) Biol. Psychiatry 44, 169–178
28. Borden, L. A. (1996) Neurochem. Int. 29, 335–356
Enkephalins Are Transported by a Novel Eukaryotic Peptide Uptake System
Melinda Hauser, Amy M. Donhardt, David Barnes, Fred Naider and Jeffrey M. Becker

J. Biol. Chem. 2000, 275:3037-3041.
doi: 10.1074/jbc.275.5.3037

Access the most updated version of this article at http://www.jbc.org/content/275/5/3037

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 5 of which can be accessed free at http://www.jbc.org/content/275/5/3037.full.html#ref-list-1