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High-efficiency synthesis of human α-endorphin and magainin in the erythrocytes of transgenic mice: A production system for therapeutic peptides

(fusion proteins/red blood cells/expression/epitopes/transgene)

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ABSTRACT **Chemical synthesis of peptides, though feasible, is hindered by considerations of cost, purity, and efficiency of synthesizing longer chains. Here we describe a transgenic system for producing peptides of therapeutic interest as fusion proteins at low cost and high purity. Transgenic hemoglobin expression technology using the locus control region was employed to produce fusion hemoglobins in the erythrocytes of mice. The fusion hemoglobin contains the desired peptide as an extension at the C end of human α-globin. A protein cleavage site is inserted between the C end of the α-globin chain and the N-terminal residue of the desired peptide. The peptide is recovered after cleavage of the fusion protein with enzymes that recognize this cleavage signal as their substrate. Due to the selective compartmentalization of hemoglobin in the erythrocytes, purification of the fusion hemoglobin is easy and efficient. Because of its compact and highly ordered structure, the internal sites of hemoglobin are resistant to protease digestion and the desired peptide is efficiently released and recovered. The applicability of this approach was established by producing a 16-mer α-endorphin peptide and a 26-mer magainin peptide in transgenic mice. Transgenic animals and their progeny expressing these fusion proteins remain healthy, even when the fusion protein is expressed at >25% of the total hemoglobin in the erythrocytes. Additional applications and potential improvements of this methodology are discussed.**

Numerous critical physiological processes require the action of peptides, including hormones and growth factors (1). For example, immune recognition is mediated by the specific binding of peptides to various receptors on T and B lymphocytes, and both cytocidal and microbicidal activities of various cells are manifested through the action of small peptides (2, 3). Much discussion has centered on the therapeutic applications of peptides, as a logical extension to their use in basic research (4). The most successful example of the pharmaceutical application of peptides so far is the development of drugs based on di- and tetrapeptide inhibitors of angiotensin-converting enzyme for the treatment of chronic hypertension. There are, however, key hindrances to the widespread use of larger peptides in human and animal medicine.

Peptide synthesis was simplified by the solid-phase synthesis methods described by Merrifield (5). The development of automated machines, novel activated amino acids, and protecting groups have led to the easy availability of peptides of various lengths. However, the cost of production of peptides remains very high and is often dictated by the primary sequence of the peptide, since certain couplings are inefficiently obtained in these machines (6). Large-scale synthesis of any peptide is further hampered by the problems of organic-solvent disposal and the purification of the desired product free of the by-products of the synthesis. For pharmaceutical applications, the removal of endotoxins and other impurities after chemical synthesis can significantly increase the cost of production. The *in vivo* circulatory half-life of peptides is short, and little is known about targeting of peptides to specific sites or cell types in the body (7). The high cost of production of therapeutic-grade peptides, combined with their poor bioavailability, has so far excluded many peptides from consideration as candidates for clinical testing. Similarly, the idea of using peptides as antigens in vaccination programs (such as for malaria) has been discussed but not yet practiced because of the large amounts of material required for these applications (8). Arguably, many of these problems could be solved by the availability of an inexpensive method for the production of peptides.

We have developed a transgenic system for peptide synthesis and purification based on the hemoglobin expression system. The desired peptide is produced as a fusion with human globin in the erythrocytes of transgenic animals. Previous reports described the efficient expression of human hemoglobin A (HbA) in mice and pigs (9–11). We have based our expression system on a class of naturally existing variant hemoglobins which contain either α- or β-globin chains extended by the addition of several amino acids (12). Both N- and C-terminal extensions have been found. These variants are expressed at moderate to low levels, are functional, and encode additional amino acids that have no known function (12). Some of these variants, especially those involving the β chain, are unstable and affect the oxygen binding affinity of hemoglobin (13). We have constructed modified α-globin genes containing DNA sequences encoding peptides of therapeutic interest. We chose α-globin as a fusion partner because of its abundant production in transgenic mice and pigs (9, 11). Hemoglobin is a compactly folded protein and in its native state is inaccessible to proteases such as trypsin (14). However, the C termini of the globin chains, particularly the α chains, are accessible to proteolytic enzymes (15). The peptide of interest can be cleaved from the fusion protein and purified by conventional methods. We chose two peptides of therapeutic interest, α-endorphin, an analgesic, and magainin, an antimicrobial, to test the validity of our expression system.

MATERIALS AND METHODS

Cloning. A 450-bp DNA fragment encoding part of the α-globin chain fused to the enterokinase cleavage site and α-endorphin was obtained by PCR and ligated to a 600-bp fragment starting after the stop codon of the α-globin gene, also obtained by PCR. The resulting fragment was linked to

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Abbreviations: IEF, isoelectric focusing; RP, reversed-phase; Hα and Hβ, human α- and β-globin; Mα and Mβ, mouse α- and β-globin.

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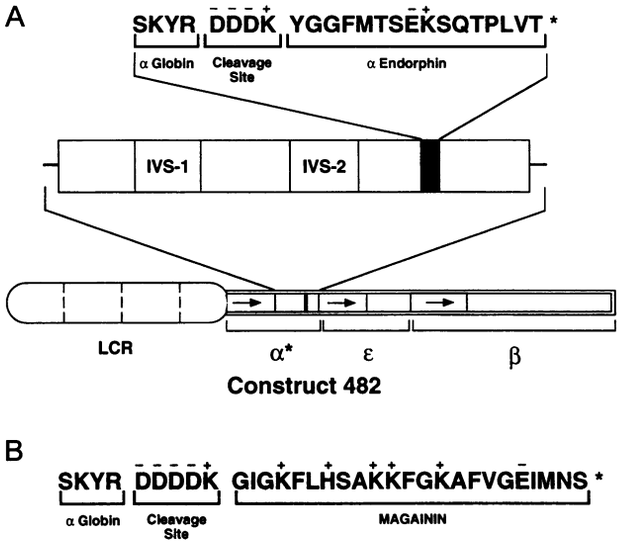

FIG. 1. Expression strategy. (*A*) Construct 482, designed to express an α-endorphin–hemoglobin fusion protein. The 16-amino acid endorphin sequence was fused to the C terminus of human α-globin. The charge of each residue is indicated. A 4-residue segment containing the cleavage site for the endoprotease enterokinase is located between the globin and the endorphin sequences. The position of introns (IVS-1 and -2) and the fused peptide sequence (black bar) are shown. This construct contains the human β-globin micro locus control region (LCR), human ε- and β-globin genes and the fusion α-globin gene (α*). (*B*) Sequence of the C-terminal region of the α-globin–magainin fusion contained in the expression vector 560. The enterokinase cleavage site, DDDDK, and the magainin sequence are shown. The charge of each residue is indicated.

an ≈1.7-kbp *Cla* I–*Hin*dIII segment of the human α-globin gene. The resultant modified α-globin gene (α*) was introduced into *Cla* I/*Kpn* I-digested pLCRεβ to get pLCRα*εβ. A 16.5-kbp fragment was obtained by *Sst* II and *Mlu* I cleavage for microinjection. The magainin expression vector was constructed similarly by using a combination of gene synthesis and PCR (Fig. 1).

Production of Transgenic Mice. DNA was injected into male pronuclei of fertilized mouse oocytes (16). Transgenic mice were identified by isoelectric focusing (IEF) of hemoglobin according to the (Isolab) instructions. The gels were fixed in 10% (wt/vol) trichloroacetic acid, photographed, and quantitated by densitometry.

Globin Chain Analysis. Hemolysates were prepared by osmotic lysis (17). The globin chains were separated from 150 μg of total hemoglobin by reversed-phase HPLC (RP-HPLC). Electrospray ionization mass spectrometry (MS) was performed with a VG Biotech (Manchester, U.K.) BIO-Q instrument with a quadrupole analyzer. Sample aliquots of 10 μl were injected into the instrument source. Elution was carried out with a 1:1 (vol/vol) methanol/water solution containing 1% acetic acid, at a flow rate of 4 μl/min.

Enterokinase Cleavage. Fusion hemoglobins were purified by elution from IEF gels. The desired proteins were eluted by diffusion into enterokinase buffer (10 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$). Enterokinase (Sigma or IBI) digestion was performed with 1.0 unit of enzyme per mg of protein in a final volume of 20 μl at 37°C for 2 hr. The digested products were analyzed by SDS/PAGE and by Triton/acid/urea electrophoresis (18).

Antibodies. Hemoglobin preparations containing human α*-globin and human β-globin (Hα*Hβ) or human α*-globin and mouse β-globin (Hα*Mβ) were purified by elution from IEF gels. Three rabbits were immunized by four injections of 75 μg of protein on days 1, 30, 45, and 60. The first injection was with complete Freund's adjuvant and subsequent injections with incomplete adjuvant. The animals were bled at days 45, 60, 90, and 105. ELISA was performed with pure hemoglobin and chemically synthesized endorphin as antigens (19).

RESULTS

Expression of Peptide–Hemoglobin Fusions. The design of the two fusion hemoglobin constructs is shown in Fig. 1. We created six transgenic mice by microinjection of construct 482 (encoding the endorphin–hemoglobin fusion) into fertilized mouse oocytes. Transgenic mice were initially identified by IEF analysis of their hemolysates. In transgenic mice expressing unmodified human α- and β-globin genes, up to 6 different types of hemoglobin can be formed: either Mαβ or $M\alpha_1M\beta$ and $M\alpha_2M\beta$ (depending on the presence of one or two α-globin genes in the mouse genome), Hαβ, Hαβ, and one or two Mαβ hybrids. Comparison with the IEF pattern of a hemolysate from a transgenic mouse expressing human HbA showed that two hemoglobins of different mobilities were present in the transgenic mice carrying construct 482. Six different bands were seen for mice 4-2 and 5-2, whereas only five bands were present for mice 4-1 and 6-5 (Fig. 2*A*). The composition of each band has been determined by RP-HPLC. This analysis revealed that hemoglobins containing the fusion α-globin (α*) protein were abundantly expressed in all six transgenic mice. Densitometric tracing of the fixed IEF gel showed that the highest expression was obtained in mouse 4-2 (Table 1), where 47% of the total hemoglobin consisted of Hα*Hβ and Hα*Mβ dimers. Expression of these two types of products varied from 8% to 46% in the other five mice (Table 1). Two founder transgenic

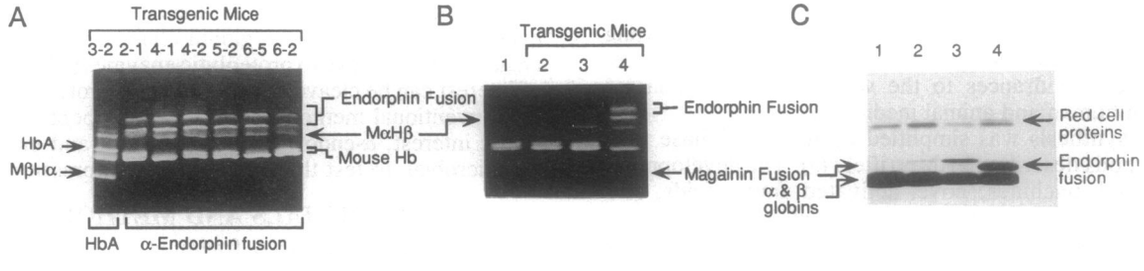

FIG. 2. Hemoglobin patterns of transgenic mice as shown by IEF in polyacrylamide gel. (*A*) Mice carrying the endorphin–hemoglobin fusion transgene. The first lane contains hemolysate from a mouse (3-2) expressing human HbA. The migration positions of human, mouse, and two interspecies hybrids are indicated. The other six lanes display hemoglobin patterns of transgenic mice carrying the endorphin fusion expression vector, construct 482. Note that the migration of fusion α-containing dimers (human and a hybrid hemoglobin) is altered compared to that seen in lane 1. (*B*) Production of a magainin fusion protein as shown by IEF analysis of hemolysates prepared from transgenic mice. The migration of various types of hemoglobin dimers is shown. Lane 1, nontransgenic mouse; lanes 2 and 3, transgenic mice containing magainin expression vector 560; lane 4, transgenic mouse expressing endorphin fusion hemoglobin. (*C*) SDS/PAGE analysis of α- and β-globin chains and the fusion globin chains.

Table 1. Expression of α-endorphin fusion hemoglobin in transgenic mice

Mouse	Expression level, %			
	Hα*Hβ	MαMβ	Hα*Mβ	HβMα
	Endorphin fusion			
2-1	8.18	74.52	10.17	7.13
4-1	21.6	41.3	26.0	11.1
4-2	26.3	44.2	19.8	9.7
5-2	14.6	71.6	6.10	7.6
6-2	4.08	88.85	4.53	2.53
6-5	11.0	70.0	13.5	5.5
	Magainin fusion†			
3-2	97.9		0.5	1.6
3-3	88.6		6.3	5.0

Expression level was determined by laser densitometric scan of IEF gels.
†For the magainin fusion, the HαHβ hemoglobin species comigrates with the MαMβ hemoglobin in the IEF gels.

mice were bred to nontransgenic mice, resulting in the generation of several progeny that expressed the fusion hemoglobin at high levels (data not shown). This high level of expression of the modified human hemoglobin in the founder as well as the second-generation transgenic mice indicates that the fusion of α-endorphin to α-globin does not destabilize the structure of the variant hemoglobin.

Two transgenic mice were created by using construct 560, containing the magainin fusion gene. These mice were bred to derive second-generation transgenic mice. IEF analysis of hemolysates prepared from these mice revealed that both the human β-globin and the fusion α-globin were expressed, as evidenced by the presence of two interspecies hybrids (Fig. 2*B*). The human fusion hemoglobin (Hα*Hβ) was not apparent from this analysis. The failure to detect the fusion protein was apparently due to the (expected) similar isoelectric points of the mouse and fusion human hemoglobins (the fusion protein is more basic than HbA). The expression of the novel globin was confirmed by SDS/PAGE (Fig. 2*C*). SDS/PAGE also provided the approximate mass of the fusion globin chains, and this mass is consistent with the anticipated extension of the α-globin.

Properties of Endorphin Fusion Hemoglobin. We have also employed an immunological strategy to probe the expression of the fusion hemoglobin. We raised antibodies to the purified endorphin fusion hemoglobin and, as a control, to native human hemoglobin in rabbits. The binding of these antibodies to chemically synthesized α-endorphin was tested by ELISA. The antisera directed against the fusion hemoglobin, but not against the normal hemoglobin, reacted with α-endorphin (Fig. 3*A*). These results suggest that the fused endorphin epitope is accessible in the fusion hemoglobin.

The functional properties of hemoglobin are influenced by various ligands that interact with the N and C termini of the globin chains as well as within the central cavity of hemoglobin (20). Fusion of the peptide to the C terminus of the α-globin might hinder the binding of these ligands to hemoglobin. Another outcome of the fusion could be the disruption of the higher-order structure of the protein, resulting in unstable or nonfunctional hemoglobin. We examined these possibilities by studying the endorphin fusion hemoglobin. First, we measured the oxygen affinity of the modified hemoglobin in transgenic blood. Blood samples drawn from mouse 4-1-2 expressing the endorphin fusion hemoglobin and an expression-level-matched control transgenic mouse, 3-1-1, expressing human HbA, were used to determine oxygen binding capacity. In these mice a pronounced "left shift" of the oxygen saturation curve (P_{50} of 21.2 and 31.8 mmHg in the test and control mice, respectively) was noticed, indicating a higher affinity of oxygen binding. Since the oxygen binding property of whole blood is influenced by the relative proportion of mouse, human, and hybrid hemoglobins, we purified human fusion hemoglobin from transgenic mouse 4-1-2 as well as HbA from human blood and measured their oxygen binding activity (Fig. 3*B*). Again, the transgenic fusion protein had higher oxygen affinity (P_{50} of 4.4) than HbA (P_{50} of 11.6 mmHg). The calculated Hill coefficient of the purified protein was lower than that of HbA (2.1 compared with 2.7), indicating a diminution in the cooperative interactions between the hemoglobin subunits.

Analysis of Fusion Globin. The authenticity of globin chains contained in the hemolysates of transgenic mice expressing HbA or the two fusion proteins was established by RP-HPLC (data not shown). In all three samples mouse α- and β-globin and the human β-globin were detected. In the HbA-expressing mouse, the human α-globin was eluted at the expected position, with an elution time of 53.31 min. No peak corresponding to normal human α-globin was found in the samples derived from mice expressing construct 482 or 560. Instead, in these samples, peaks presumably corresponding to the modified α-globins were eluted at 49.16 min (endorphin fusion) and 51.86 min (magainin fusion). We isolated these two peaks by preparative RP-HPLC and determined their respective mass by electrospray MS. Both samples gave strong positive-ion mass spectra which showed major components of 17327.4 Da (calculated mass, 17326.285) and 18162.1 Da (calculated mass, 18162.168) in the endorphin and magainin fusion globins, respectively. These values are very close (within 1.1 and .06 Da, respectively) to the calculated mass of the fusion α-globins, suggesting that no posttranslational modification of these chains occurred within the red cells.

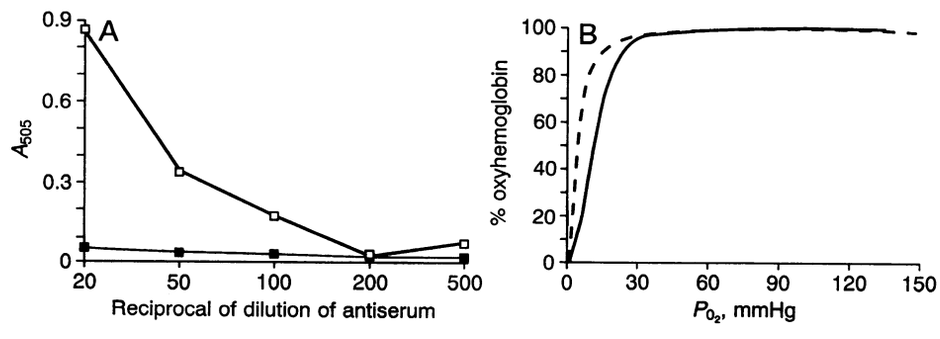

FIG. 3. Properties of endorphin fusion hemoglobin. (*A*) Immunodetection of endorphin. Rabbits were immunized with HbA (■) or the endorphin fusion hemoglobin (□) and the antisera were tested by ELISA for binding to synthetic α-endorphin. (*B*) Oxygen affinity measurements. HbA and the endorphin fusion hemoglobin were purified by preparative IEF. Oxygen equilibrium curves of HbA (solid line) and fusion hemoglobin (broken line) are shown. The P_{50} values and Hill coefficients (n) are as follows: HbA, P_{50} = 11.6 mmHg and n = 2.7; fusion hemoglobin, P_{50} = 4.4 mmHg and n = 2.1 (1 mmHg = 133 Pa).

Enterokinase Cleavage. We tested the feasibility of peptide recovery by enterokinase digestion of fusion hemoglobin present in the transgenic hemolysate. No cleavage sites for enterokinase, a highly specific endoprotease (21), are present in human or mouse α- and β-globins. Aliquots of hemolysates from normal mouse and transgenic mice expressing the two peptides were incubated with enterokinase. The effect of this digestion was assessed by SDS/PAGE of samples before and after digestion (Fig. 4). No changes were detected in the 16-kDa globin chains of HbA. On the other hand, the fusion α globin was completely cleaved, as evidenced by the disappearance of the 18- or 19-kDa band in the digested samples (Fig. 4). Triton/acid/urea denaturing gel electrophoresis (data not shown) revealed that the unmodified β chain remained intact and only the fusion α chain was cleaved. Rapid digestion was also observed when purified hemoglobin was used instead of crude hemolysate as a substrate (data not shown). These results demonstrate that the enterokinase cleavage strategy is successful and that the C terminus of the fusion α-globin is readily accessible to enterokinase in the hemoglobin tetramer, as is the case for other proteases such as trypsin and carboxypeptidase (15).

Recovery of the Fused Peptide. The quantitative release and recovery of the designed peptide in a limited number of steps are critical for the efficient production of a therapeutic peptide as a fusion protein. The above studies reflect the presence of the designed peptide, but the integrity of the released peptide was not confirmed by these experiments. Accordingly, we digested hemoglobin in lysates prepared from transgenic mice expressing endorphin and magainin as fusion proteins. Surprisingly, the digested endorphin fusion sample did not contain a peak corresponding in elution time to the chemically synthesized endorphin. Instead, this sample contained two early peaks not seen in the digested hemolysate from transgenic mice expressing HbA or in undigested transgenic hemolysate (Fig. 5). To understand the origin of these new peaks, chemically synthesized endorphin was also subjected to enterokinase digestion under identical conditions. When resolved by HPLC, this digested sample revealed the same two peaks seen upon digestion of the fusion hemoglobin with the enzyme. This suggests that an unanticipated site of enterokinase cleavage is present in the endorphin sequence. In the endorphin sequence (Fig. 1), there is an EK dipeptide which is partly related to the canonical enterokinase cleavage site DDDDK. Presumably, enterokinase has cleaved both at the junction of α-globin and the peptide as well as within the endorphin. Such degenerate specificity has previously been described for this enzyme (21) and points to the need for the careful design of the cleavage site. Degenerate cleavage may also be overcome by careful control of the digestion conditions.

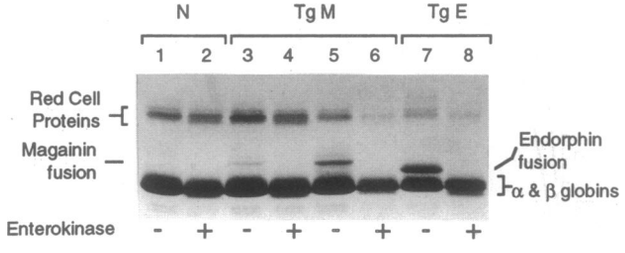

FIG. 4. Enterokinase digestion. Hemolysates prepared from blood samples of nontransgenic (N) and transgenic mice expressing magainin or endorphin fusion proteins (Tg M and Tg E, respectively) were incubated with enterokinase for 16 hr at 25°C. Samples taken before (−) or after (+) digestion were resolved by SDS/PAGE. The positions of α-globin–magainin fusion (lanes 3 and 5), α-globin–endorphin fusions (lane 7), nonglobin red-cell proteins (seen in all lanes), and α- and β-globins (seen in all lanes) are indicated.

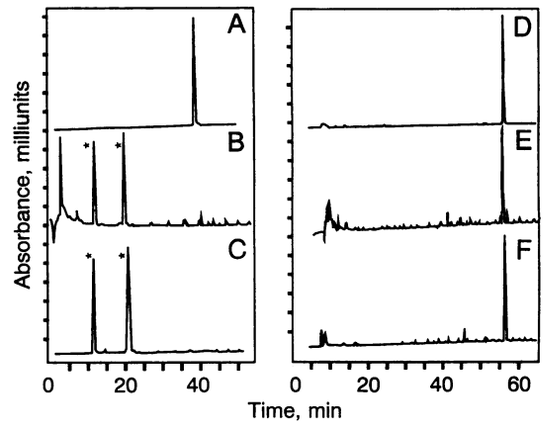

FIG. 5. Recovery of peptides by enterokinase cleavage. The relevant portions of the RP-HPLC elution patterns are shown. (*A*) Synthetic α-endorphin. (*B*) Enterokinase-digested hemolysate from transgenic mice expressing endorphin fusion. The asterisks indicate two small peptides released upon digestion. (*C*) Enterokinase-digested synthetic endorphin. (*D*) Synthetic magainin. (*E*) Enterokinase-digested hemolysate of a transgenic mouse expressing magainin fusion. (*F*) Enterokinase-digested purified magainin fusion hemoglobin.

We also digested the magainin fusion hemoglobin with enterokinase. The magainin sequence does not contain any DK or EK sequences. The digested globin released a peptide that corresponded in elution with the synthetic magainin (Fig. 5). No other small peptides were recovered. Similar digestion patterns were obtained when either the crude hemolysate or the IEF-purified hemoglobin was used as a substrate. The released peptide was collected and subjected to amino acid sequencing to confirm the identity of the released magainin. These results validate the hemoglobin fusion approach for the production of peptides in transgenic animals.

DISCUSSION

The methodology of chemical synthesis of peptides has been refined to a stage where peptides of 100 or more residues can be synthesized (22). Microbial expression of fusion proteins has been widely practiced but poses problems of unintended modification, endotoxins, and purification. The technology described in this paper is likely to lower the cost of synthesis of peptides, especially when large-scale production is desired. High yields (>1 g/liter) of peptides of 10 or more residues can be obtained even at modest expression levels that are easily achievable in transgenic animals (11). Peptides such as magainin, whose production in large quantities at a low cost may be required, are particularly suited for transgenic expression. Production can be increased further by several means. The replacement of an endogenous globin gene with the fusion gene by homologous recombination can potentially increase expression (to 100%), provided that a functional hemoglobin molecule can be constituted with the fusion globin chains. It may be possible to fuse in tandem two or more copies of the peptide sequence, ensuring a greater fractional yield of the peptide. Finally, it is conceivable that for certain sequences, hemoglobin containing both α- and β-globin fusions can be engineered.

What is the longest peptide chain that can be incorporated within hemoglobin? We have shown here that extensions of 20 and 30 amino acids are efficiently produced as fusion α-globins. Although this number is likely to be sequence dependent, up to 31 additional residues are found in naturally existing elongated α-globin chains (12). An α–α fusion, a "dialpha globin," is stable and can be made by recombinant expression systems (ref. 23 and unpublished data). In the

transgenic expression system, high-level expression of fusion globin depends not only on the proper synthesis of the protein but also on the functional properties of the modified hemoglobin. The endorphin fusion hemoglobin has a higher affinity for oxygen than HbA. It is known that the oxygen affinity of hemoglobin is increased when the C termini are amidated (24). High-affinity hemoglobin variants are widespread in nature and in some cases their expression portends higher accumulation of the mutant allele (25). All the mice carrying the fusion proteins appear to be healthy, including those that are third-generation transgenic. The lack of an adverse phenotype in our mice may indicate either that the fused peptides are inactive or that the peptides are sequestered inside the erythrocytes, away from their site of action. Magainins have a tendency to insert into membranes, causing cytolysis (3). No unusual hemolysis or cytopathology was observed in transgenic mice expressing the magainin fusion, but further hematological and biochemical analyses are required to confirm the subcellular location of this fusion protein.

The enterokinase cleavage site is likely to be widely useful, since it is rare and efficient and can introduce a net negative charge on the fusion protein. There is anecdotal evidence suggesting that an increase in the acidity of the globin chain may be beneficial for its high-level production (26). The enterokinase signal requires five to six additional amino acids, and for the synthesis of longer peptides the use of shorter signals, such as those for cyanogen bromide or hydroxylamine cleavage, may be desirable. The C-terminal residue of α-globin, arginine, can be used for trypsin or ArgC-mediated cleavage when the peptide sequence lacks arginine and/or lysine residues. Our findings suggest that a dipeptide, EK, is a functional recognition site for enterokinase. It is remarkable that, under the conditions of digestion used here, none of the potential EK and DK sites within the globin chains were digested by enterokinase. This suggests that the folded hemoglobin molecule excludes the enzyme (a feature that is of great practical importance for the recovery of the peptide). Only peptides that do not require unnatural or modified amino acids can be synthesized in this system. Certain sequences may not be suitable for expression by this method, because of structural or size constraints. The fusion hemoglobin, when immunogenic, can be used as a vaccine. Hemoglobin itself is a poor immunogen but may serve as a carrier of desired epitopes. Finally, certain peptides and proteins could be fused to globins with the intent of making hemoglobin more suitable for use as a cell-free oxygen carrier.

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