Thorpe, T. J. N. Carber, L. Groucutt, L. J. Kricka, *Nature* **305**, 158 (1983)] with the use of a chemiluminescence kit (Amersham) according to the vendor's instructions. In resting cells, a total of 41 distinct bands were recognized, ranging from 17 to 200 kD. IL-1 induced an increase in phosphorylation of a select subset within 5 min, including proteins with the following molecular weights: 27, 44, 78, 101, 110, 122, and 160 kD. Sphingomyelinase and C8-cer similarly induced phosphorylation of this set of proteins.

30. Incubations were terminated with $CHCl_3$:CH_3OH:HCl (100:100:1) containing 15 mM EDTA. Lipids in the organic-phase extract were dried under nitrogen and subjected to mild alkaline hydrolysis (0.1 M methanolic potassium hydroxide for 1 hour at 37°C) to remove glycerophospholipids. Sphingomyelin was resolved by thin-layer chromatography (TLC) with $CHCl_3$:CH_3OH:CH_3COOH:H_2O (60:30:8:5) as solvent, identified by iodine vapor staining, and quantified by liquid scintillation spectrometry [R. N. Kolesnick and A. Paley, *J. Biol. Chem.* **262**, 9204 (1987)].
31. J. Preiss *et al.*, *ibid.* **261**, 8597 (1986); K. A. Dressler and R. N. Kolesnick, *ibid.* **265**, 14917 (1990).
32. After treatment with IL-1, cells were homogenized at 4°C with a Dounce homogenizer in buffer [25 mM Hepes (pH 7.4), 5 mM EGTA, 50 mM NaF, leupeptin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml)]. Homogenates were centrifuged at 500*g* for 5 min to remove nuclei and at 200,000*g* for 30 min to prepare microsomal membranes. Membranes were resuspended in homogenizing buffer (2.2 μg of membrane protein per microliter). For assay of kinase activity, the reaction mixture contained 20 μl of microsomal membrane, 40 μl of EGFR peptide [4 mg/ml in 25 mM Hepes (pH 7.4)], and 100 μl of buffer [50 mM Hepes (pH 7.4) and 20 mM $MgCl_2$] (*3*). Phosphorylation was initiated at 22°C by the addition of 40 μl of [γ-^{32}P]ATP (100 μM final concentration) and terminated at the indicated times by the addition of 40 μl of 0.5 M ATP in 90% formic acid. Phosphorylated peptide was eluted from a C_{18} Sep pak cartridge (Millipore), lyophilized, and resolved by C_{18} reversed-phase high-pressure liquid chromatography with a linear gradient of acetonitrile. The peptide eluted at 30% acetonitrile, as determined by measurement of Cerenkov radiation in 1-ml fractions. Baseline kinetic analyses revealed a maximum reaction velocity (V_{max}) of 12.5 pmol min^{-1} mg^{-1} of microsomal membrane protein and Michaelis constants (K_m) of 70 μM for ATP and 0.15 mg/ml for EGFR peptide. For most studies, 100 μM ATP was used to maintain a high ^{32}P-specific radioactivity (4000 dpm/pmol), although qualitatively similar results were obtained with 500 μM ATP. Ceramide and sphingosine (10 nM to 1 μM) enhanced kinase activity to 1.4- to 2.5-fold of control values.
33. We thank M. Moore for the recombinant human IL-1β. Supported by grants RO-1-CA-42385 from the National Cancer Institute of the National Institutes of Health and FRA-345 from the American Cancer Society (R.N.K.), the Charles A. Dana Foundation (A.Y.), and the Sloan-Kettering Institute Clinical Scholar Award for Biomedical Research (A.Y.).

30 July 1992; accepted 2 November 1992

Regulation by Heme of Mitochondrial Protein Transport Through a Conserved Amino Acid Motif

Julia Tait Lathrop and Michael P. Timko*

A conserved motif, termed the heme regulatory motif (HRM), was identified in the presequences of the erythroid δ-aminolevulinate synthase precursors and was shown to be involved in hemin inhibition of transport of these proteins into mouse mitochondria in vitro. When the HRM was inserted into the presequence of the ornithine transcarbamoylase precursor, a normally unregulated mitochondrial protein, it conferred hemin inhibition on the transport of the chimeric protein. The conserved cysteine within the HRM was shown by site-directed mutagenesis to be required for hemin inhibition.

Heme has a variety of catabolic and regulatory functions within cells. It serves as a prosthetic group or cofactor for numerous hemoproteins and coenzymes (*1*, *2*), maintains the initiation of translation of certain eukaryotic mRNAs (*3*, *4*), and controls the DNA binding capabilities of some transcription factors (*5*). Heme also regulates its own synthesis through feedback inhibition of transcription and transport of δ-aminolevulinic acid synthase (ALAS; E.C. 2.3.1.37), the first enzyme of the heme biosynthetic pathway. ALAS catalyzes the condensation of glycine and succinyl–coenzyme A to form the first intermediate of the pathway, δ-aminolevulinic acid, a reaction that is localized to the mitochondrial matrix in all animal cells. Murine ALAS is synthesized as an approximately 70-kD cytosolic precursor (preALAS) that is post-translationally transported into the mitochondrial matrix, where it is proteolytically processed to form a 65-kD mature enzyme. Three isoforms of ALAS are expressed in animals. A housekeeping form, generally referred to as the hepatic form (ALAS-H), is in the biosynthetic pathway of heme for basic cellular functions and is regulated by heme in response to changing cellular heme requirements. Two developmentally regulated erythroid forms, ALAS-E major and ALAS-E minor, are involved in the formation of heme for hemoglobin. The ALAS-E minor form, which accounts for 15% of the total ALAS-E mRNA, arises from an alternative splicing event that deletes 45 bp in exon 3 (*6*). The deletion alters neither the translation frame nor the catalytic domain of the protein but eliminates 15 amino acids that are present in the NH_2-terminus of the mature ALAS-E major protein.

Exogenous hemin can regulate the amount of preALAS-H mRNA (*7*). High hemin concentrations can also inhibit the transport of preALAS-H into mitochondria both in vivo (*8*, *9*) and in vitro (*10*). The developmentally regulated ALAS-Es are expressed exclusively in differentiating erythroid tissue, and their translation is coordinated with cytosolic iron levels through an iron responsive element (IRE) in the 5′ untranslated region of the mRNA (*11*). Studies of differentiation-inducible cell lines and human reticulocytes have implicated heme in posttranscriptional regulation as well (*12*). We describe here the identification and characterization of a heme regulatory motif (HRM) in the mouse ALAS-E precursors (preALAS-E major and preALAS-E minor) that confers sensitivity to feedback inhibition by hemin of mitochondrial transport of these preproteins. We demonstrate that this HRM could confer hemin inhibition of transport on a mitochondrial protein that is normally unregulated.

Most precursors of mitochondrial proteins destined to be transported from the

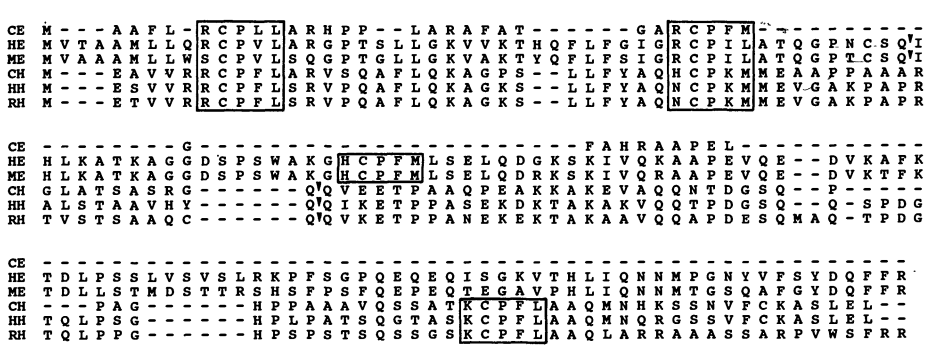


Fig. 1. Comparison of the amino acid composition of chick, human, and mouse erythroid (CE, HE, and ME) and chick, human, and rat hepatic (CH, HH, and RH) ALAS presequences (*29*). The conserved motifs (HRMs) are boxed. The arrowheads indicate the processing site within the various precursors.

Department of Biology, University of Virginia, Charlottesville, VA 22901.

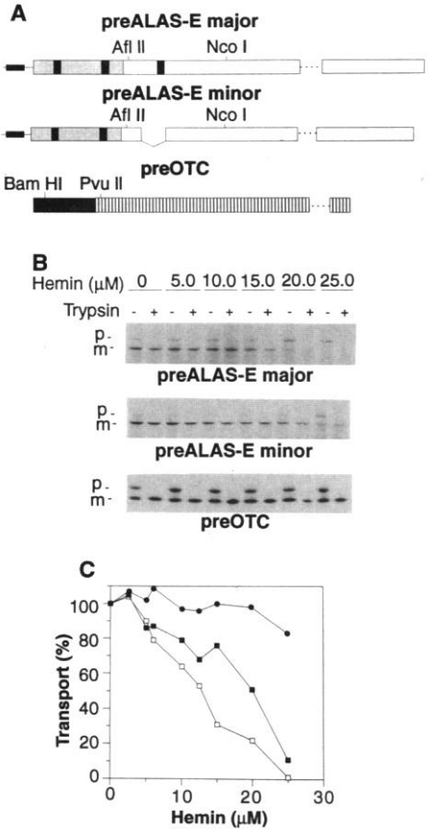
*To whom correspondence should be addressed.

cytoplasm into the matrix are synthesized with NH_2-terminal amphiphilic presequences that direct the protein into the mitochondrion (*13*). Although the presequences of the housekeeping or erythroid forms of preALAS from chick, human, mouse, and rat are greater than 80% identical, the preALAS-E and preALAS-H presequences are only 24% identical when compared to each other (Fig. 1). The amino acid residues in common among the preALAS-H and preALAS-E presequences are contained primarily within two copies of a conserved motif. This motif [(Arg, Lys, or Asn)-Cys-Pro-, either Lys or a hydrophobic residue, Leu or Met, where the cysteine and proline are invariant], we term the HRM. PreALAS-E major contains a third copy of the HRM in the NH_2-terminus of the mature protein (Fig. 1) that is eliminated from the preALAS-E minor form by differential splicing (Fig. 2A). The HRM present in the preALASs is similar to a consensus sequence required for heme activation of the yeast transcription factor HAP1 (*5*) and to sites present in other hemoproteins, including hemopexin and catalase (*14, 15*).

To investigate the potential role of the HRMs, we cloned the cDNAs that encoded the preALAS-E major and preALAS-E minor proteins into pBluescript plasmids, and the cDNAs were transcribed in vitro and the mRNAs were translated in cell-free wheat germ extracts that had been optimized to control hemin concentrations in our mitochondrial transport reactions (*16*). Transport of the preALAS-E major and preALAS-E minor proteins into isolated mouse mitochondria was inhibited 99% and 91%, respectively, in the presence of 25 μM exogenous hemin (Fig. 2, B and C). The transported mature proteins were properly compartmentalized because externally added protease did not degrade them (Fig. 2B). In contrast, transport of the precursor to human mitochondrial ornithine transcarbamoylase (preOTC) (*17*), assayed under identical reaction conditions, was not inhibited at low hemin concentrations and was inhibited less than 20% by 25 μM hemin (Fig. 2, B and C). In experiments where the preALAS-E and preOTC were translated and transported in the same reaction, the response of the respective precursors to hemin inhibition was maintained (*18*), which suggests that the sensitivity of transport to the amount of hemin was a property inherent to the individual protein.

To investigate the role of the preALAS-E presequence in hemin inhibition of transport, we constructed chimeric proteins that linked the presequence and a portion of the mature peptide (encompassing the region containing the 15–amino acid deletion in the preALAS-E minor form) to the mature OTC protein (*19*) (Fig. 3A). Transport of preALAS-E major–OTC (which contains three copies of the HRM) and preALAS-E minor–OTC (which contains two HRMs) was inhibited 89% and 87%, respectively, by 25 μM hemin (Fig. 3, B and C), an inhibition similar to that observed for the native preALAS-Es.

Internal deletions made within the presequence of the chimeric proteins were used to identify the sequences involved in the observed hemin inhibition. These deletion studies revealed that a region of the presequence that contained a single HRM (that most proximal to the NH_2-terminus) was sufficient to mediate hemin inhibition of transport (*18*). It was not possible, however, for us to unambiguously distinguish between loss of hemin inhibition and loss of transport capability by deleting this HRM because of its location within the first 15 amino acids of the presequence (residues 11



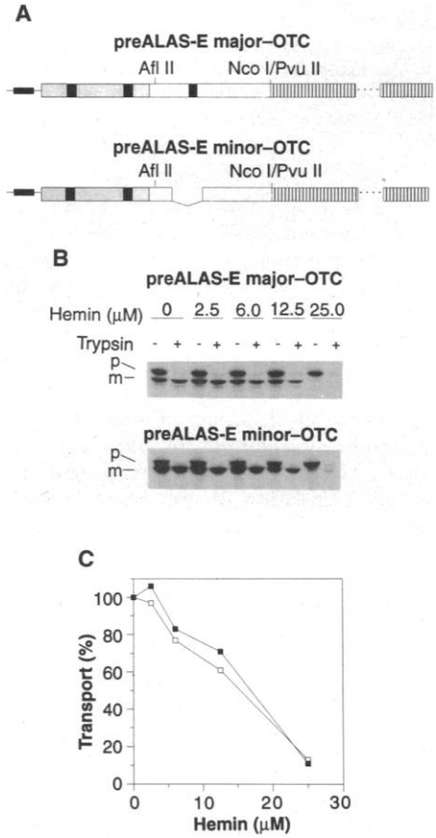


Fig. 2 (left). Hemin inhibition of preALAS-E transport into mouse mitochondria. (**A**) Structure of the coding sequences for the three precursors used in these experiments. In preALAS-E major and minor, the coding sequence for the mature protein is indicated by a white box, the presequence by light gray, the HRM by black, and the IRE by a thick black line. The location of the 15–amino acid deletion in preALAS-E minor is indicated by the bent line. In the preOTC, the coding sequence for the mature protein is indicated by vertical hatches, and the presequence is indicated by a dark gray box. (**B**) Representative autoradiograph of [^{35}S]methionine-labeled proteins after transport in the absence or presence of added hemin. The cDNAs for the various constructs were transcribed, and the mRNA was translated in vitro (*16*). Transport reactions were done in the presence of the indicated hemin concentrations for 1 hour at 25°C (*30*), the mitochondria were precipitated, and half of each sample (+) was incubated for 10 min at room temperature with TPCK-treated trypsin (5 μg/ml) (Sigma; TPCK, L-1-tosylamido-2-phenylether chloromethyl ketone). Both treated and untreated samples were subjected to 7.5% SDS–polyacrylamide gel electrophoresis, dried, fluorographed with 1 M salicylic acid, and exposed to preflashed Kodak-XAR film. Shown are transport reaction products before (−) and after (+) treatment with TPCK-treated trypsin. Precursor protein, p; mature protein, m. (**C**) Quantitation of hemin inhibition of transport for the various proteins in the experiment shown (*30*); □, preALAS-E major; ■, preALAS-E minor; and ●, preOTC. **Fig. 3 (right).** Hemin inhibition of the transport of chimeric proteins into mouse mitochondria. (**A**) Structure of the coding sequences for the precursors of the chimeric proteins used in these experiments. Structural designations are the same as those in Fig. 2. (**B**) Representative autoradiograph of [^{35}S]methionine-labeled proteins after transport in the absence or presence of added hemin; p and m are as in Fig. 2. (**C**) Quantitation of hemin inhibition of transport for the various proteins; □, preALAS-E major–OTC; ■, preALAS-E minor–OTC. Transport assays and quantitation were conducted as in Fig. 2.

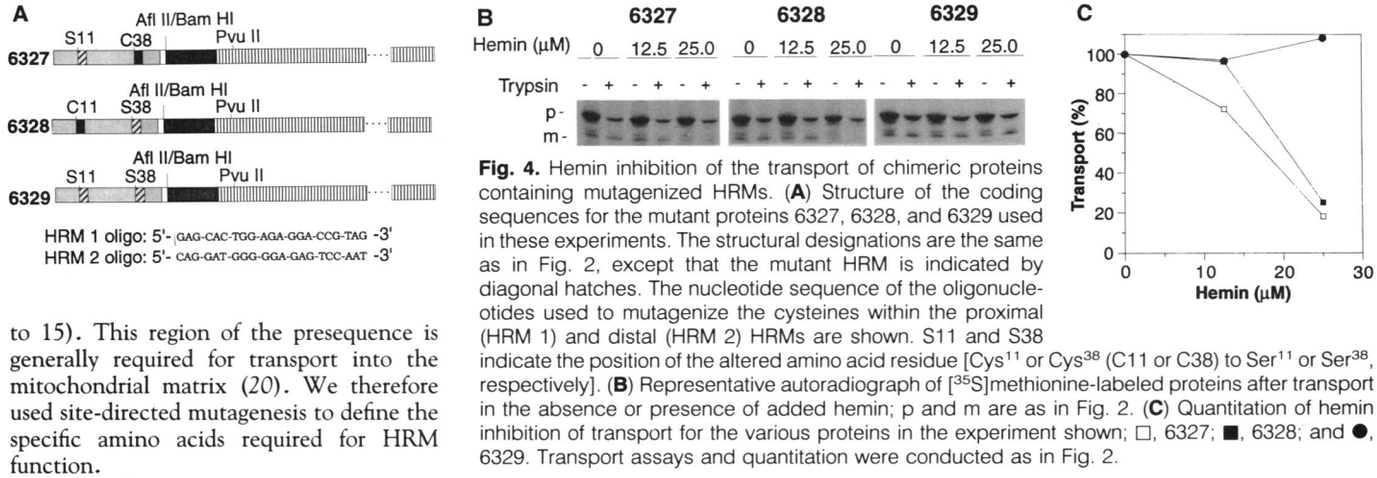


Fig. 4. Hemin inhibition of the transport of chimeric proteins containing mutagenized HRMs. (**A**) Structure of the coding sequences for the mutant proteins 6327, 6328, and 6329 used in these experiments. The structural designations are the same as in Fig. 2, except that the mutant HRM is indicated by diagonal hatches. The nucleotide sequence of the oligonucleotides used to mutagenize the cysteines within the proximal (HRM 1) and distal (HRM 2) HRMs are shown. S11 and S38 indicate the position of the altered amino acid residue [Cys^{11} or Cys^{38} (C11 or C38) to Ser^{11} or Ser^{38}, respectively]. (**B**) Representative autoradiograph of [^{35}S]methionine-labeled proteins after transport in the absence or presence of added hemin; p and m are as in Fig. 2. (**C**) Quantitation of hemin inhibition of transport for the various proteins in the experiment shown; □, 6327; ■, 6328; and ●, 6329. Transport assays and quantitation were conducted as in Fig. 2.

to 15). This region of the presequence is generally required for transport into the mitochondrial matrix (*20*). We therefore used site-directed mutagenesis to define the specific amino acids required for HRM function.

Conserved cysteine residues are often involved in the coordination of heme to its apoprotein (*2*). To explore the involvement of the cysteines in the HRMs in hemin inhibition, we mutagenized the cysteines to serines by a single nucleotide change (*19*) (Fig. 4A). Replacement of cysteine in either the proximal or distal HRM resulted in a 75 to 82% inhibition of transport by 25 μM hemin (Fig. 4, B and C). Replacement of both cysteines by serines eliminated the hemin inhibition of transport (Fig. 4, B and C). To determine whether a single HRM could confer hemin-regulated transport to a normally unregulated protein, we synthesized an HRM that contained either cysteine or serine and inserted it into the human preOTC presequence (Fig. 5A). Transport of preOTC(Cys^{10}) was inhibited 86% by 25 μM hemin, whereas preOTC(Ser^{10}) showed the same insensitivity to hemin inhibition observed in the native preOTC (Fig. 5, B and C).

Thus, our data suggest that feedback inhibition operates at two different points after transcription to regulate ALAS-E activity. Translation of the ALAS mRNA appears to be coupled to the concentration of free iron through the action of an IRE (*11*). We show that exogenous hemin regulates transport of preALAS-E into the mitochondrion and identify a conserved amino acid motif (HRM) as the element mediating this feedback inhibition. A single HRM can regulate the transport of preALAS-E or a normally unregulated protein into the mitochondrion.

Cytosolic free heme concentrations have been estimated to rise to 100 μM during the early stages of erythroid differentiation (*21*). Thus, the relatively high concentrations of hemin required for nearly complete transport inhibition in vitro are within physiological levels for a developing erythrocyte. As globin synthesis is induced by heme (*22*) through its general effect on translation (*3*, *4*), heme feedback inhibition of ALAS-E transport and therefore activity could serve as a mechanism to fine-tune the very tight coordination of heme synthesis with globin apoprotein production during erythropoiesis.

The conserved cysteine residue within the HRM appears to be required for its function. The mechanism of interaction of heme with preALAS-E at these residues is unknown. Non-HRM cysteines present in the presequences of the preOTC and the chimeric proteins 6329 and preOTC(Ser^{10}) are not capable of mediating hemin inhibition of transport. Because mitochondrial precursors must be unfolded to be competent for transport (*23*), direct interaction of preALAS-E with heme through the HRM could alter the conformation of the protein or its interaction with the transport machinery, rendering it incapable of moving through the outer membranes. The conservation of the HRM in the highly diverged presequences of both the erythroid and hepatic isoforms of the ALAS precursors suggests that transport inhibition of the hepatic isoform may be mediated through the HRM in a manner similar to that of preALAS-E. The presence of the HRM in other proteins (*5*, *14*), in particular the heme-activated transcription factor HAP1, suggests that HRM-mediated heme interaction with proteins may be a general mechanism for the control of synthetic and regulatory processes within the cell.

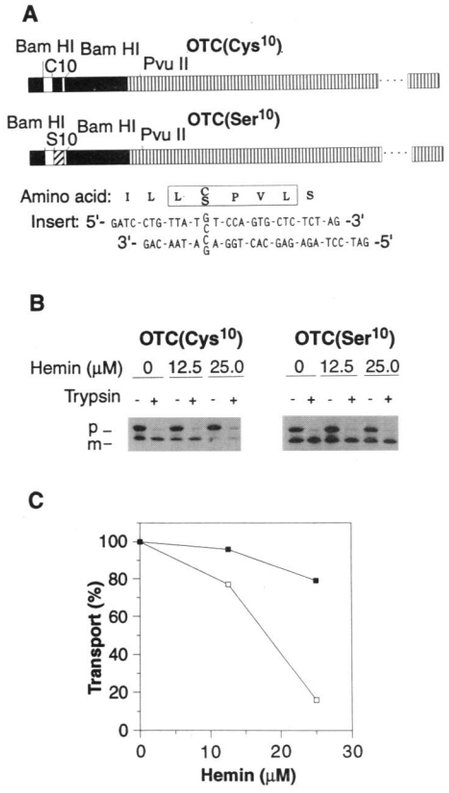


Fig. 5. Hemin inhibition of transport of preOTC containing an HRM insertion. (**A**) Structure of the coding sequences for the mutant proteins used in these experiments. The structural designations are the same as in Fig. 4, except that the insert containing the HRM is indicated by a white box. The nucleotide sequence of oligonucleotides used to create the insert containing the synthetic HRM with either a Cys^{10} (C10) or a Ser^{10} (S10) residue is shown. The amino acid sequence of the synthetic HRM is boxed (*29*). (**B**) Representative autoradiograph of [^{35}S]methionine-labeled proteins after transport in the absence or presence of added hemin; p and m are as in Fig. 2. (**C**) Quantitation of hemin inhibition of transport for the various proteins in the experiment shown; □, preOTC(Cys^{10}); ■, preOTC(Ser^{10}). Transport assays and quantitation were conducted as in Fig. 2.

REFERENCES AND NOTES

1. R. J. Kulmacz and W. E. M. Lands, *J. Biol. Chem.* **259**, 6358 (1984).
2. D. M. Hampsey, G. Das, F. Sherman, *ibid.* **261**, 3259 (1986); Y. Tanaka, I. Kubota, T. Amachi, H. Yoshizumi, H. Matsubara, *J. Biochem.* **108**, 7 (1990).

3. J.-J. Chen, J. M. Yang, R. Petryshyn, N. Kowower, I. M. London, *J. Biol. Chem.* **264**, 9559 (1989).
4. A. I. Grayzel, P. Hörchner, I. M. London, *Proc. Natl. Acad. Sci. U.S.A.* **55**, 650 (1966); I. M. London *et al.*, in *The Enzymes*, P. D. Boyer and E. G. Krebs, Eds. (Academic Press, New York, 1987), vol. 18, pp. 359–380.
5. K. Pfeifer, K.-S. Kim, S. Kogan, L. Guarente, *Cell* **56**, 291 (1989).
6. D. S. Schoenhaut and P. J. Curtis, *Nucleic Acids Res.* **17**, 7013 (1989); E. G. Young, thesis, University of Virginia (1990).
7. G. Srivastava, J. D. Brooker, B. K. May, W. H. Elliot, *Biochem. J.* **188**, 781 (1980); P. D. Drew and I. Z. Ades, *Biochem. Biophys. Res. Commun.* **162**, 102 (1989); J. W. Hamilton *et al.*, *Arch. Biochem. Biophys.* **289**, 387 (1991).
8. G. Srivastava *et al.*, *Biochem. Biophys. Res. Commun.* **117**, 344 (1983).
9. K. Yamauchi, N. Hayashi, G. Kikuchi, *J. Biol. Chem.* **255**, 1746 (1980).
10. N. Hayashi, N. Watanabe, G. Kikuchi, *Biochem. Biophys. Res. Commun.* **115**, 700 (1983).
11. P. Ponka and H. M. Schulman, *J. Biol. Chem.* **260**, 14717 (1985); T. C. Cox, M. J. Bawden, A. Martin, B. K. May, *EMBO J.* **10**, 1891 (1991); T. Dandekar *et al.*, *ibid.*, p. 1903.
12. C. Beaumont *et al.*, *Exp. Cell Res.* **154**, 474 (1984); L. C. Gardner, S. J. Smith, T. M. Cox, *J. Biol. Chem.* **266**, 22010 (1991).
13. D. Roise and G. Schatz, *J. Biol. Chem.* **263**, 4509 (1988); K. Verner and G. Schatz, *Science* **241**, 1307 (1988); K. P. Baker and G. Schatz, *Nature* **349**, 205 (1991).
14. H. Nikkilä, J. D. Gitlin, U. Muller-Eberhard, *Biochemistry* **30**, 823 (1991).
15. B. L. Triggs-Raine, B. W. Doble, M. R. Mulvey, P. A. Sorby, P. C. Loewen, *J. Bacteriol.* **170**, 4415 (1988).
16. Experimental constructs, cloned into the pBluescript (Stratagene) plasmids, were transcribed with T3 RNA polymerase under standard reaction conditions in the presence of $m^7(5')Gppp(5')G$. The mRNAs were translated in the presence of [^{35}S]methionine (Trans-^{35}S label, 1066 Ci/mmol, ICN Radiochemicals) in a wheat germ extract (Promega) treated for 30 min at 4°C with *N*-acetyl-D-glucosamine attached to agarose beads (Sigma). Protein synthesis was stopped by the addition of 30 μM cycloheximide, and the translation products were frozen in liquid nitrogen and stored at −80°C until use. Mice (DBA/2) were cervically dislocated and exsanguinated, and mitochondria were isolated from the livers (*24*). Transport assays were done as described (*25*) with the following modifications. Transport reactions (25 μl final volume) contained freshly isolated mitochondria (100 μg of total protein) in 5 μl of isolation buffer (*24*), 10 μl of untreated rabbit reticulocyte lysate (Promega) (*26*), and 5 μl of [^{35}S]methionine-labeled proteins in buffer containing 12 mM Hepes (pH 7.6), 0.5 mM spermidine, creatine phosphokinase (50 μg/ml), 10 μM creatine phosphate, 25 mM potassium acetate, 1 mM magnesium acetate, and 5 mM dithiothreitol. Hemin (Porphyrin Products, Logan, UT) prepared as described (*27*) was added in a final volume of 5 μl at the indicated concentrations. Hemin concentrations were determined as described (*28*). Animal care was in accordance with institutional guidelines. Experiments were repeated a minimum of three times with preALAS-E major, preALAS-E minor, and preOTC as controls, and all results were reproducible under all conditions tested.
17. A. L. Horwich *et al.*, *Science* **224**, 1068 (1984).
18. J. T. Lathrop, thesis, University of Virginia (1992).
19. For the construction of the preALAS-E–OTC fusion proteins, plasmid encoding the preOTC was digested with Bam HI, Xho I, and Pvu II. The 1044-bp fragment encoding the mature OTC was ligated into the preALAS-E vectors prepared by digestion with Nco I, blunted with Klenow fragment of DNA polymerase I, and digested with Bam HI. The resulting fusion proteins contained the 92 NH_2-terminal residues of preALAS-E linked to the COOH-terminal 318 amino acids of mature OTC. For the construction of the mutant proteins 6327, 6328, and 6329, plasmid encoding preOTC was digested with Bam HI, blunted, and digested with Pst I. The 1350-bp fragment was ligated into preALAS-E vectors. The ALAS-E HRMs were mutagenized with the oligonucleotides shown in Fig. 4 according to the Altered Sites technical manual (Promega). The mutagenized plasmids were prepared by digestion with Afl II, blunted, and digested with Bam HI. The resulting fusion proteins contain the 53 NH_2-terminal residues of preALAS-E linked to the 402 COOH-terminal amino acids of preOTC.
20. T. Keng, E. Alani, L. Guarente, *Mol. Cell. Biol.* **6**, 355 (1986); G. Schatz, *Eur. J. Biochem.* **165**, 1 (1987).
21. S. C. Lo, R. Aft, G. C. Mueller, *Cancer Res.* **41**, 864 (1981).
22. J. Ross and D. Sautner, *Cell* **8**, 513 (1976).
23. M. Eilers, S. Hwang, G. Schatz, *EMBO J.* **7**, 1139 (1988); W.-J. Chen and M. G. Douglas, *J. Biol. Chem.* **262**, 15605 (1987).
24. C. Schnaitman and J. W. Greenawalt, *J. Cell Biol.* **38**, 158 (1968).
25. J. G. Conboy and L. E. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3073 (1981).
26. K. Murakami, Y. Amaya, M. Takiguchi, Y. Ebina, M. Mori, *J. Biol. Chem.* **263**, 18437 (1988).
27. R. S. Ranu and I. M. London, *Methods Enzymol.* **60**, 459 (1979).
28. K. M. Smith, Ed., in *Porphyrins and Metalloporphyrins* (Elsevier/North-Holland, New York, 1975), pp. 804–807.
29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. Protein import into mitochondria was linear under the described experimental conditions (*16*) for 90 min. The amount of transport at 1 hour was determined by densitometry of autoradiographs with the use of the Adobe Photoshop (Adobe Systems, Mountfairview, CA) 2.0 and Image 1.40b3 computer programs (W. Rashand, National Institute of Mental Health). Densitometry was standardized, and measurements were taken in the linear range as determined by standard curves. Graphs present mean values for replicated experiments. Representative autoradiographs are presented.
31. We thank P. Dierks, in whose laboratory this project was initiated, for stimulating discussions, L. Rosenberg for the gift of the preOTC clone, and B. Lathrop and R. Umek for their critical reading of the manuscript. Supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (5 RO1 DK33304-06) and NSF (MCB-9005308).

22 July 1992; accepted 3 November 1992

Identification of the SH3 Domain of GAP as an Essential Sequence for Ras-GAP–Mediated Signaling

Marc Duchesne,* Fabien Schweighoffer,* Fabienne Parker, François Clerc, Yveline Frobert, Ming Nguy Thang, Bruno Tocqué†

Guanosine triphosphatase activating protein (GAP) is an essential component of Ras signaling pathways. GAP functions in different cell types as a deactivator and a transmitter of cellular Ras signals. A domain (amino acids 275 to 351) encompassing the Src homology region 3 (SH3) of GAP was found to be essential for GAP signaling. A monoclonal antibody was used to block germinal vesicle breakdown (GVBD) induced by the oncogenic protein Ha-ras Lys^{12} in *Xenopus* oocytes. The monoclonal antibody, which was found to recognize the peptide containing amino acids 275 to 351 within the amino-terminal domain of GAP, did not modify the stimulation of the Ha-Ras–GTPase by GAP. Injection of peptides corresponding to amino acids 275 to 351 and 317 to 326 blocked GVBD induced by insulin or by Ha-Ras Lys^{12} but not that induced by progesterone. These findings confirm that GAP is an effector for Ras in *Xenopus* oocytes and that the SH3 domain is essential for signal transduction.

Ras proteins are required for the functioning of the signal transduction pathways that regulate cell growth and differentiation (*1*, *2*). The function of Ras in mammalian cells depends on its interaction with GAP, a protein that can stimulate the intrinsic GTPase activity of Ras (*3*). A complex between Ras and GAP is responsible for interrupting muscarinic atrial K^+ channel currents (*4*, *5*). In Chinese hamster ovary cells Ras induces transcription from the polyoma virus enhancer only in the presence of full-length GAP (*6*). Mutations in Ras (in the so-called effector domain between amino acids 30 and 40) block biological activity and GAP-stimulated GTP hydrolysis (*7*, *8*). GAP binds to a number of proteins in vitro. The COOH-terminal domain is sufficient for binding to cellular Ras proteins (*9*). The NH_2-terminal domain contains SH2 and SH3 domains that allow

M. Duchesne, F. Schweighoffer, F. Parker, F. Clerc, B. Tocqué, Rhone Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, 13 Quai Jules Guesde, B.P. 14, 94403 Vitry Sur Seine, France.
Y. Frobert, Service de Pharmacologie et d'Immunologie, DRIPP Centre d'Etudes de Saclay, CEA, 91191 Gif-Sur-Yvette, France.
M. N. Thang, Inserm U245, Hopital St Antoine, 184 Rue Faubourg St Antoine, 75571 Paris Cédex 11, France.

*Contributed equally to this manuscript.
†To whom correspondence should be addressed.