paired nucleotides which in turn are preceded by four stem-loop structures. The AUG initiator codon itself also overlaps part of a predicted stem-loop structure.
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28. 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.
29. We thank M. W. Carter for excellent technical assistance and M. A. Pickett for advice on automated DNA sequencing.

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Activation of the Sphingomyelin Signaling Pathway in Intact EL4 Cells and in a Cell-Free System by IL-1β

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The mechanism of interleukin-1 (IL-1) signaling is unknown. Tumor necrosis factor–α uses a signal transduction pathway that involves sphingomyelin hydrolysis to ceramide and stimulation of a ceramide-activated protein kinase. In intact EL4 thymoma cells, IL-1β similarly stimulated a rapid decrease of sphingomyelin and an elevation of ceramide, and enhanced ceramide-activated protein kinase activity. This cascade was also activated by IL-1β in a cell-free system, demonstrating tight coupling to the receptor. Exogenous sphingomyelinase, but not phospholipases A_2, C, or D, in combination with phorbol ester replaced IL-1β to stimulate IL-2 secretion. Thus, IL-1β signals through the sphingomyelin pathway.

Hydrolyis of sphingomyelin to ceramide at the plasma membrane by a neutral sphingomyelinase may initiate a cascade that functions in signaling (*1*–*6*). Ceramide may stimulate a Ser-Thr kinase termed ceramide-activated protein kinase to transduce the signal (*2*–*4*). Ceramide-activated protein kinase is membrane-bound, Mg^{2+}-dependent, and defined by its capacity to phosphorylate a synthetic peptide (amino acids 663 to 681) derived from the amino acid sequence surrounding Thr^{669} of the epidermal growth factor receptor (EGFR). Ceramide-activated protein kinase may be a member of an emerging family of proline-directed Ser-Thr kinases that includes the extracellular signal-regulated (also referred to as mitogen-activated) and $p34^{cdc2}$ kinases (*7*). Substrates for these kinases contain the minimal recognition sequence, X-Ser/Thr-Pro-X, in which the phosphorylated site is flanked on its COOH-terminus by a proline residue and X can be any amino acid.

Tumor necrosis factor (TNF)–α may use the sphingomyelin pathway for signaling (*3*, *4*, 6). TNF stimulates this pathway early during HL-60 cell differentiation into monocytes, and synthetic ceramide analogs bypass receptor activation and directly induce differentiation (*4*–6). This cascade can be reconstituted in a cell-free system comprised of extracts of HL-60 cells, which demonstrates tight coupling of this pathway to the TNF receptor (*4*). The present studies were performed because of numerous reports that TNF and IL-1 stimulate a common set of events in diverse biologic systems (*8*).

The murine thymoma EL4 cell line is a well-defined IL-1–responsive cell line that expresses functional IL-1 receptors (*9*, *10*). Upon stimulation with IL-1, these cells up-regulate the IL-2 receptor and secrete IL-2 (*10*). Initial studies were designed to investigate the effects of IL-1β on cellular sphingomyelin content. Cells grown in Dulbecco's modified Eagle's (DME)–Ham's F-12 medium containing 10% horse serum and [^{3}H]choline (1 μCi/ml) were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β. Under these conditions, IL-1β induced time- and concentration-dependent sphingomyelin hydrolysis (Fig. 1, A and B). A maximally effective concentration of IL-1β (40 ng/ml) induced a detectable reduction by 2 min in sphingomyelin content from a baseline of 800 ± 14 pmol per 10^6 cells (mean ± SEM) to 648 ± 16 pmol per 10^6 cells ($P < 0.005$) (*11*) at 30 min. Concentrations of IL-1β of 0.01 ng/ml were effective, with a maximal effect at 10 ng/ml [effective dose (ED_{50}) ~ 2 ng/ml (Fig. 1B)]. A similar reduction in sphingomyelin content after IL-1 stimulation was determined by direct measurement of phosphorus content (*12*). In contrast, the content of phosphatidylcholine, the other major choline-containing phospholipid, was unchanged.

Under the same conditions, IL-1β in-

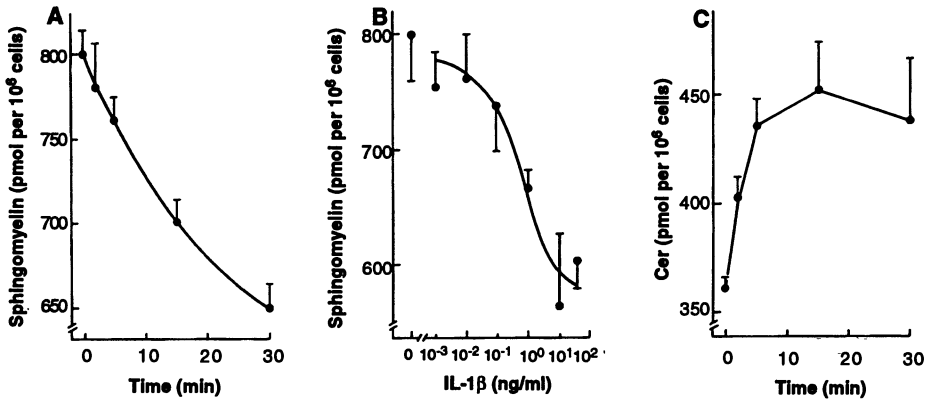

Fig. 1. IL-1β effects on sphingomyelin and ceramide in EL4 cells. (**A** and **B**) Sphingomyelin time course (A) and dose response (B). (**C**) Ceramide time course. Cells were grown to growth arrest (1 to 1.5 × 10^6 cells per milliliter) in DME–Ham's F12 medium (1:1, v/v) containing 10% horse serum. For sphingomyelin measurements, [^{3}H]choline (1 μCi/ml) was added 48 hours before an experiment. The use of [^{3}H]choline as a measure of sphingomyelin content was validated by simultaneous phospholipid phosphorus measurements (*12*). On the day of an experiment, cells were resuspended in the same medium at 10 × 10^6 cells per milliliter and stimulated with IL-1β (40 ng/ml) for the indicated times (A and C) or for 30 min with increasing concentrations of IL-1β (B). After extraction of lipids, sphingomyelin was resolved by TLC (*30*). Ceramide was quantified enzymatically with the use of the *Escherichia coli* diacylglycerol kinase reaction as described (*31*). Each value represents the mean ± SEM of triplicate determinations from four experiments in (A), one representative of four similar studies performed in triplicate in (B), and triplicate determinations from ten experiments in (C).

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duced a statistically significant increase in ceramide content (Fig. 1C). Ceramide increased from 360 to 403 pmol per 10^6 cells at 2 min ($P < 0.005$) and to a maximum of 450 pmol per 10^6 cells at 15 min. In separate studies ($n = 4$), a statistically significant increase in ceramide content was evident by 30 s. Maximally effective concentrations of other agents known to stimulate EL4 cells (*13*, *14*), including 12-O-tetradecanoylphorbol-13-acetate (TPA), concanavalin A, epinephrine, and an antibody to CD3 did not elicit a ceramide response ($n = 5$). Hence, sphingomyelinase activation appeared specific for stimulation by IL-1β.

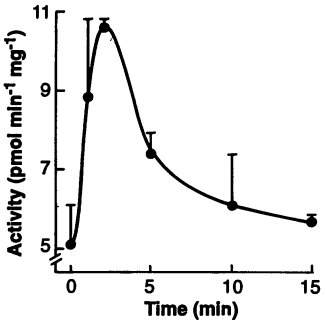

Fig. 2. Effect of IL-1β on ceramide-activated protein kinase activity. Cells (30 × 10^6 per milliliter) treated as in Fig. 1 were stimulated with IL-1β (10 ng/ml). Microsomal membrane was used for assay of kinase activity, with EGFR peptide as a substrate (*32*). Phosphorylated peptide was resolved by high-performance liquid chromatography. All assays were performed under linear conditions, as determined for time and enzyme concentration. Enzyme activity was determined from the percentage conversion of substrate to product and the specific radioactivity of [γ-^{32}P]ATP. Values (mean ± range) represent duplicate determinations from two experiments.

Subsequent studies assessed whether IL-1β also enhanced ceramide-activated protein kinase activity. EL4 cells contained a membrane-bound, ceramide-activated protein kinase activity similar to that reported in A431 human epidermoid carcinoma cells and HL-60 cells (*3*, *4*). Activity was measured by the transfer of ^{32}P from the γ-position of adenosine triphosphate (ATP) to the EGFR peptide. The effect of IL-1β on kinase activity was determined with the use of microsomal membranes derived from cells stimulated with IL-1β. IL-1β enhanced kinase activity in a time- and concentration-dependent manner. In cells treated with 10 ng/ml of IL-1β, an increase in kinase activity was detectable at 30 s and maximal at 2 min (Fig. 2, $P < 0.005$). Activity increased to 2.1-fold of control values, from 5 to 11 pmol min^{-1} mg^{-1}, and then gradually declined over 15 min. Concentrations of IL-1β of 0.03 ng/ml were effective, with a maximal effect at 10 ng/ml ($ED_{50} \sim 2$ ng/ml). This is the same range of concentrations effective for sphingomyelin hydrolysis. Stimulation by IL-1β was detected in a total of ten experiments. Cytosolic fractions of EL4 cells also contained kinase activity toward EGFR peptide of 2.6 ± 0.3 (mean ± range) pmol min^{-1} mg^{-1}. Cytosolic activity, which represents proline-directed protein kinase activities other than ceramide-activated protein kinase (*15*), was not enhanced by IL-1β during these studies. Further, protein kinase C activity, as determined by phosphorylation of lysine-rich histone (type III-S) (*16*), was not enhanced in either membrane or cytosolic fractions.

Table 1. Induction of IL-2 secretion by IL-1 and sphingomyelinase. EL4 cells (1.5 × 10^6 per milliliter) were treated with IL-1β (10 ng/ml), sphingomyelinase (SMase) (*Staphylococcus aureus*), PLA_2 (*Vipera russelli*), PLC (*Bacillus cereus*), and PLD (*Streptomyces chromofuscus*) at the indicated concentrations in the absence or presence of TPA (20 ng/ml). Boiled sphingomyelinase preparations had no activity. Culture supernates were harvested at 24 hours and assayed for secreted IL-2 with the use of a mouse IL-2 ELISA kit (Genzyme Corp.) according to the manufacturer's instructions. The lower limit of sensitivity of this assay was 15 pg of IL-2, and the assay was linear up to 960 pg of IL-2. Data (mean ± range) represent duplicate determinations from two experiments. ND, not detectable.

Cell treatment	IL-2 secretion (pg)	
	Diluent	TPA
Control	ND	ND
IL-1β (10 ng/ml)	ND	261 ± 2
SMase (1×10^{-3} U/ml)	ND	313 ± 60
PLA_2 (1 to 5×10^{-2} U/ml)	ND	ND
PLC (1 to 5×10^{-2} U/ml)	ND	ND
PLD (1 to 5×10^{-2} U/ml)	ND	ND

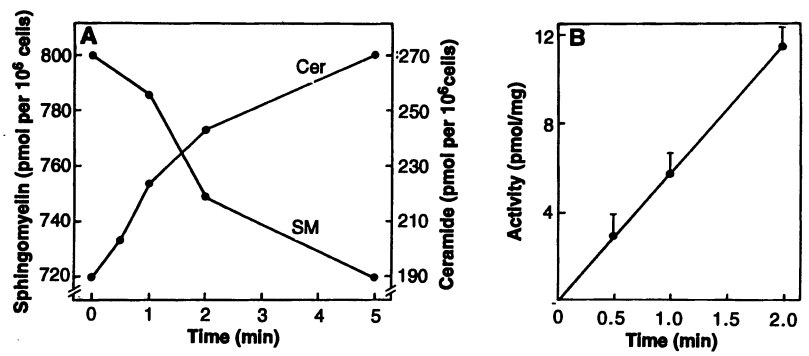

Fig. 3. IL-1β effects on sphingomyelin and ceramide concentrations (**A**) and ceramide-activated protein kinase activity (**B**) in a cell-free system. Nuclei-free supernates prepared as in Fig. 2 were incubated for 10 min at 4°C with IL-1β (10 ng/ml) or diluent (DME:F12 with 10% horse serum) to allow for ligand-receptor interaction. Thereafter, supernates (300 μg per incubation in 25 μl) were added to a reaction mixture (total volume of 250 μl) as in Fig. 2. For studies measuring lipid, incubations were stopped by extraction of lipids into an organic phase and resolved as in Fig. 1. For studies measuring kinase activity, incubations contained EGFR peptide and [^{32}P]ATP, and phosphorylated peptide was quantified as in Fig. 2. Background activity was subtracted from each point. Values (mean) represent data from two experiments for sphingomyelin performed in triplicate, three experiments for ceramide performed in triplicate, and five experiments for ceramide-activated protein kinase activity performed in duplicate.

Early activation of a potential signaling system supports the notion that the pathway might be involved in the signaling process. However, signal transduction pathways are tightly regulated and often interrelated (*17*). Hence, activation of one system often results in rapid activation of another. To provide additional support for tight coupling of the sphingomyelin pathway to activation of the IL-1β receptor, we studied signaling in subcellular fractions of EL4 cells. For these studies, supernates collected after a low-speed centrifugation to remove nuclei were first incubated with IL-1β for 10 min at 4°C to permit formation of IL-1–receptor complexes (*4*). Thereafter, we initiated reactions by warming supernates to 22°C in a reaction mixture containing Mg^{2+} at pH 7.4. We adopted these conditions to allow for activation of endogenous neutral sphingomyelinase (*18*). For studies measuring kinase activity, reaction mixtures also contained [^{32}P]ATP and EGFR peptide. Under these conditions, IL-1β stimulated a rapid reduction in sphingomyelin content and a quantitative increase in ceramide content (Fig. 3A). In separate studies, a statistically significant reduction in sphingomyelin content ($n = 10$) and elevation in ceramide content ($n = 6$) were detected at 1 min of stimulation ($P < 0.005$ versus control). Ceramide-activated protein kinase activity also increased (Fig. 3B). These effects were quantitatively

similar to those determined in the intact cells. Hence, activation of the sphingomyelin pathway by IL-1β was also observed in a cell-free system.

To determine whether the sphingomyelin pathway mediated the biologic response to IL-1β, we compared direct activation of the sphingomyelin pathway by exogenous sphingomyelinase (*4*) with stimulation by IL-1β. For these studies, cells were treated with IL-1β, sphingomyelinase, and phorbol ester, and IL-2 secreted into the media after 24 hours was measured. As reported (*10, 13, 19*), IL-1β (1 to 30 ng/ml) or TPA (1 to 20 ng/ml) alone did not induce detectable IL-2 secretion (Table 1). However, IL-1β (10 ng/ml) and TPA (20 ng/ml) in combination stimulated maximal secretion. Similarly, sphingomyelinase alone did not stimulate IL-2 secretion, but in combination with TPA induced secretion. Concentrations of sphingomyelinase between 5×10^{-5} and 1×10^{-1} U/ml were effective, and 1×10^{-3} U/ml of sphingomyelinase stimulated maximal secretion ($ED_{50} \sim 7 \times 10^{-4}$ U/ml). In separate studies ($n = 2$), sphingomyelinase (1×10^{-3} U/ml) induced secretion at all concentrations of TPA, from 0.5 to 20 ng/ml. This concentration of sphingomyelinase induced an increase in ceramide content quantitatively similar to that induced by maximally effective concentrations of IL-1β and has previously been shown to mimic TNF action in HL-60 cells (*4*). In contrast, phospholipases (PL) A_2, C, and D at concentrations 10 to 50 times higher than maximally effective sphingomyelinase did not stimulate IL-2 secretion alone or in combination with TPA. Hence, the costimulation of IL-2 secretion by IL-1 and TPA in EL4 cells was mimicked specifically by activation of the sphingomyelin pathway with sphingomyelinase.

In additional studies ($n = 3$), the ability of exogenous ceramide to stimulate IL-2 secretion was assessed. A cell-permeable synthetic ceramide analog, C8-ceramide (C8-cer), has previously been used to directly activate the kinase (*2, 3*). C8-cer alone, like IL-1β and sphingomyelinase, did not induce IL-2 secretion. However, C8-cer (0.1 to 10 μM) added in four doses at 2-hour intervals costimulated IL-2 secretion with TPA. Maximal IL-2 secretion to 270 ± 21 pg/ml occurred at 1 μM ceramide after 48 hours ($ED_{50} \sim 0.3$ μM).

Although signaling for IL-1 has been ascribed to various protein kinases (including protein kinases A and C) and a Ser-Thr protein kinase (*20*), no coherent picture has emerged to account for all of the data. Two distinct IL-1 receptors of 60 and 80 kD have been cloned (*21*). These receptors are homologous in their extracellular ligand binding domains but have little homology in their cytoplasmic portions. In fact, the 60-kD receptor has only a short intracellular portion. There is no empiric or structural evidence suggesting that these receptors themselves might serve as protein kinases (*21*). In addition, these receptors are not homologous to any protein known to be involved in signal transduction. The present studies define the mechanism by which the IL-1 receptor might activate a protein kinase. Whether this pathway is involved in signaling for IL-1 in other systems needs to be tested. However, preliminary studies with the human natural killer–like cell line, YT (*22*), demonstrate that IL-1 also induces rapid generation of ceramide in this system.

Despite the reported similarities in the action of TNF-α and IL-1, primary sequence homology between their receptors is limited. Hence, the mechanism by which these two cytokines activate the sphingomyelin signal transduction pathway is not readily apparent. The ability to reconstitute this pathway in a cell-free system and the availability of immunoprecipitating (*23*) and activating or inactivating (*23, 24*) antibodies to these receptors and the neutral sphingomyelinase (*25*) should permit investigations into the mechanism involved in these interactions.

In sum, these studies provide evidence that the effects of IL-1β may be mediated by the sphingomyelin signal transduction pathway. In this paradigm, ligand binding to the receptor activates a neutral sphingomyelinase, hydrolyzing sphingomyelin to ceramide. Neutral sphingomyelinase appears to be ubiquitous in mammalian cells and like sphingomyelin may be externally oriented in the plasma membrane (*26*). Colocalization of receptor, phospholipase, and substrate at the plasma membrane suggests that ceramide will be generated at this site. Ceramide, which can redistribute across a lipid bilayer, then stimulates a ceramide-activated protein kinase that phosphorylates a specific subset of cellular proteins, thereby altering their function (*27–29*).

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27. A ceramide-activated protein phosphatase has recently been described [R. T. Dobrowsky and Y. A. Hannun, *J. Biol. Chem.* **267**, 5048 (1992)]. This activity was exclusively cytosolic and was inhibited by okadaic acid. Although the role of this phosphatase in signaling was not studied, the cytosolic location suggests that it acts after events at the plasma membrane. These data are consistent with the notion that ceramide itself might direct a phosphorylation-dephosphorylation cascade.
28. During the preparation of this revision, L. R. Ballou *et al.* [*J. Biol. Chem.* **267**, 20044 (1992)] reported that sphingomyelin hydrolysis may mediate, in part, the effect of IL-1 on the activation of human dermal fibroblasts.
29. Although IL-1 action might be initiated through a Ser-Thr kinase, tyrosine kinases are rapidly recruited in the early stages of signaling [G. R. Guy *et al.*, *J. Biol. Chem.* **266**, 14343 (1991)]. Similarly, in EL4 cells, IL-1 induced a unique pattern of protein tyrosine phosphorylation. For these studies, cells were treated with optimal concentrations of IL-1 (10 ng/ml), C8-cer (1 to 10 μM), or sphingomyelinase (1×10^{-3} U/ml). Equal quantities of protein obtained after lysis with NP-40 were electrophoresed as described [C. K. Joseph *et al.*, *J. Biol. Chem.* **267**, 1327 (1992)]. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) by electroblotting, incubated with an anti-phosphotyrosine antibody (UBI), and visualized by an enhanced chemiluminescence system [T. P. Whitehead, G. H. G.

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)].
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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.).

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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

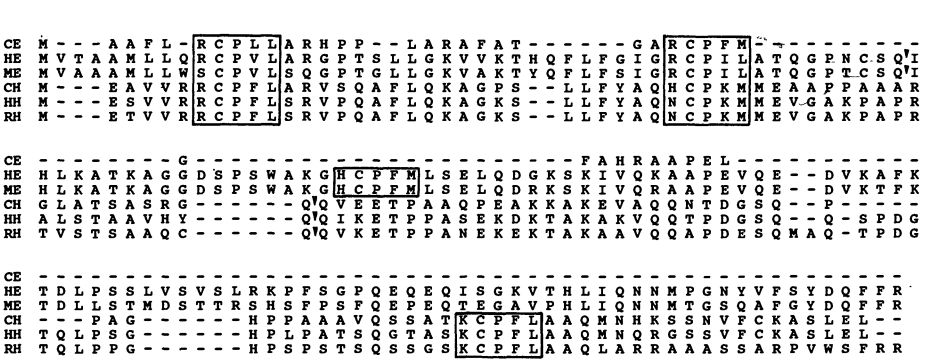

Fig. 1. Comparison of the amino acid composition of chick, human, and mouse erythroid (CE, HE, and ME) and chick, human, and rat hepatic (CH, HH, and RH) ALAS presequences (*29*). The conserved motifs (HRMs) are boxed. The arrowheads indicate the processing site within the various precursors.

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