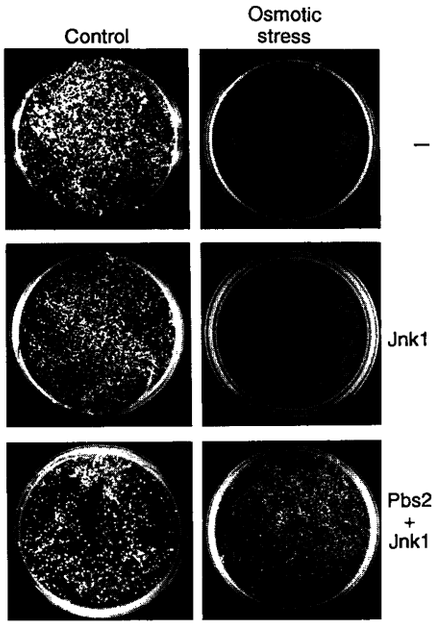

Fig. 4. Function of Jnk1 within an osmosensing signal transduction pathway requires the MAP kinase kinase Pbs2. Yeast lacking the protein kinases Hog1 (*hog1-Δ1*) and Pbs2 (*hog1-Δ1 pbs2-Δ1*) were transformed with expression vectors and spread on agar plates supplemented without (control) or with (osmotic stress) 0.9 M NaCl. Photographs of representative plates showing the growth of yeast colonies are presented (*16*).

sion in osmotically shocked cells has been established. Examples include the system A amino acid transporter (*2*) and the Na^+-inositol cotransporter (*3*). Thus, Jnk may regulate transcription factors that control the expression of these genes. The transcription factor c-Jun is known to bind to Jnk and to be phosphorylated at two sites within the NH_2-terminal activation domain (*8*, *9*), resulting in increased transcriptional activity (*11*). Furthermore, osmotic shock induces the expression of c-Jun (*12*) and, in some tissues, c-Fos (*13*). Increased activity of the transcription factor AP-1 may therefore be relevant to the osmotic shock response. Indeed, Jnk could participate in this process by activating AP-1 or other transcription factors.

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14. Chinese hamster ovary cells were co-transfected with the plasmids pCMV-Flag-Jnk1 and pRSV-Neo (*8*). A stable cell line expressing epitope-tagged Jnk1 (Flag; Immunex, Seattle, WA) was isolated by selection with Geneticin (Gibco-BRL). The cells were incubated with different concentrations of sorbitol for defined times at 37°C. The cells were collected in lysis buffer [20 mM tris (pH 7.4), 1% Triton X-100, 2 mM EDTA, 137 mM NaCl, 25 mM β-glycerophosphate, 1 mM orthovanadate, 2 mM pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml)], and a soluble extract was obtained by centrifugation at 100,000*g* for 30 min at 4°C. The epitope-tagged Jnk1 was isolated by immunoprecipitation with the monoclonal antibody M2 (Immunex). The immunoprecipitates were washed extensively with lysis buffer. Immunecomplex kinase assays were done in 25 μl of 25 mM Hepes (pH 7.4), 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 100 μM orthovanadate, and 50 μM [γ-^{32}P] ATP (10 Ci/mmol) with 2.5 μg of bacterially expressed c-Jun (residues 1 to 79) fused to glutathione-S-transferase as a substrate (*8*).
15. Single-letter abbreviations for the amino acid residues are as follows: 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.
16. The Hog1 expression vector pJB17 and the yeast strains JBY10 (null mutant *hog1-Δ1*) and JBY1041 (null mutant *hog1-Δ1 pbs2-Δ1*) were obtained from M. Gustin (*5*). Human Erk2 [F. A. Gonzalez, D. L. Raden, M. R. Rigby, R. J. Davis, *FEBS Lett.* **304**, 170 (1992)] and Jnk1 (*8*) complementary DNAs have been described. Mutations of Jnk1 were constructed as described [S. N. Ho *et al.*, *Gene* **77**, 51 (1989)]. Expression vectors for Jnk1 and Erk2 were constructed by sub-cloning of the complementary DNAs at the Bam HI and Hind III sites of pVP16 [A. B. Vojtek, S. M. Hollenberg, J. A. Cooper, *Cell* **74**, 205 (1993)]. The structure of all plasmids was confirmed by sequencing with an Applied Biosystems (Foster City, CA) model 373A machine. The yeast were transformed with the plasmids, and equal portions were spread on agar plates supplemented without and with 0.9 M NaCl as described (*5*).

17. We thank M. Gustin for providing yeast strains and plasmids, C. Peterson for advice about complementation assays, T. Barrett for DNA sequencing, and M. Shepard for excellent secretarial assistance. We also thank M. Dickens, S. Gupta, and H. Sluss for discussions. Supported by a grant from the National Cancer Institute. R.J.D. is an Investigator of the Howard Hughes Medical Institute.

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A MAP Kinase Targeted by Endotoxin and Hyperosmolarity in Mammalian Cells

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Mammalian cells respond to endotoxic lipopolysaccharide (LPS) by activation of protein kinase cascades that lead to new gene expression. A protein kinase, p38, that was tyrosine phosphorylated in response to LPS, was cloned. The p38 enzyme and the product of the *Saccharomyces cerevisiae HOG1* gene, which are both members of the mitogen-activated protein (MAP) kinase family, have sequences at and adjacent to critical phosphorylation sites that distinguish these proteins from most other MAP kinase family members. Both HOG1 and p38 are tyrosine phosphorylated after extracellular changes in osmolarity. These findings link a signaling pathway in mammalian cells with a pathway in yeast that is responsive to physiological stress.

Few extracellular stimuli elicit systemic responses in mammals that are as immediate and profound as those resulting from exposure to LPSs of Gram-negative bacteria. LPSs are endotoxins. They are prototypical activators of cells of the immune and inflammatory systems and initiate the systemic changes known as septic shock (*1*). Cells of monocytic origin [monocytes and macrophages (MOs)] respond to LPS by releasing mediators that produce septic shock (*2*). CD14, a glycosylphosphatidylinositol-anchored membrane glycoprotein of MOs, serves as an LPS receptor controlling cell activation under physiological conditions (*3*, *4*).

Binding of LPS to CD14 causes rapid protein tyrosine phosphorylation of a number of intracellular proteins that in some, but not all, LPS-responsive cells include the 44- and 42- or 40-kD isoforms of the mitogen-activated protein kinases (MAPK1 and MAPK2, respectively) (*5*, *6*). LPS also

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induces protein tyrosine phosphorylation of a 38-kD protein, p38, that appears to be distinct from MAPK1 and MAPK2 (*7*). Here we report on purification, cloning, and sequencing of complementary DNA (cDNA) encoding p38, together with an analysis of the properties of p38. The sequence of p38 shows that it is a member of the MAPK family with features in its primary sequence that are similar to those of the product of the *Saccharomyces cerevisiae* osmosensing gene *HOG1* (*8*) and that distinguish it from most other known members of the MAPK family. We found that, like its counterpart in yeast, p38 was also tyrosine phosphorylated in response to increases in extracellular osmolarity.

A murine pre–B cell line, 70Z/3, was transfected with human CD14. The tyrosine phosphorylated form of p38 was isolated from LPS-treated 70Z/3-hCD14 cells by antiphosphotyrosine affinity chromatography (*9*). Peptide sequence information was used to prepare a specific nucleotide probe that we used to screen a murine liver cDNA library. A cDNA clone encoding p38 was isolated, and both strands of DNA were sequenced (*10*) and shown to encode a 360–amino acid 41-kD protein (GenBank accession number U10871). Northern (RNA) blot analysis showed that a 3.5-kb mRNA encoding p38 was present in primary isolates of murine MOs and of B and T lymphocytes, as well as in MO-like, T, B, and fibroblast cell lines (*11*). Immunoblotting showed that recombinant p38 expressed in *Escherichia coli* was tyrosine phosphorylated because it was recognized by an antibody to phosphotyrosine, and in vitro kinase assays showed that recombinant p38 was enzymatically active—it phosphorylated myelin basic protein, IκBα, and itself, but not bovine serum albumin (*11*).

A database search (*12*) showed that p38 shares sequence identity with the yeast MAPK family member encoded by the *HOG1* gene (*8*) and with other mammalian MAPKs, including murine MAPK2 (Fig. 1). The predicted amino acid sequence of p38 has 52% identity with that of HOG1 and from 46 to 49% identity with the sequences of a group of MAPK family members that includes MAPK1 and MAPK2 (49%), SPK1 (49%), FUS3 (46%), and KSS1 (46%) (*12*). Like HOG1, p38 contains the conserved amino acid sequences (domains I through XI) found in protein kinases, including the MAPKs (*13*). The Thr^{180} and Tyr^{182} residues in p38 just before subdomain VIII (highlighted by a black box in Fig. 1) are in a comparable position in all MAPKs; phosphorylation of these residues is required for enzymatic activity (*14*). The kinases p38 and HOG1 share a TGY (*15*) sequence, whereas most other MAPK family members are characterized by a TEY (*15*) sequence (*14*). JNK1, a distant relative of the MAPK group, contains a TPY (*15*) sequence in the dual phosphorylation site (*16*). From the three-dimensional structure of MAPK2, the TEY sequence is seen to be part of a structure termed linker L12, the loop between protein kinase subdomains VII and VIII (*17*). The corresponding loop of p38 and HOG1 (*8*) is six amino acids shorter, which suggests that phosphorylation of p38 and HOG1 may occur by mechanisms distinct from those that control activation of MAPK family members with TEY sequences. In support of this idea are data showing that HOG1 phosphorylation is mediated by a specific MAP kinase kinase, PBS2, rather than by other yeast MAP kinase kinases (*8*). Despite the sequence similarity among p38, HOG1, and other MAPK family members, the deletion of six amino acids in a potentially critical region and the presence of the TGY sequence lead us to suggest that p38 and HOG1 may be members of a distinct group of MAPK-related enzymes.

We introduced cDNA encoding p38 into a yeast expression vector, pAB23BXN (*18*), to determine if p38 would complement a HOG1 mutation (*8*). Yeast transformants were tested for growth in the presence of 0.9 M NaCl (Fig. 2) (*8*). Expression of p38 in a *HOG1* mutant strain (*hog1-Δ1*) complemented yeast that failed to grow on medium supplemented with 0.9

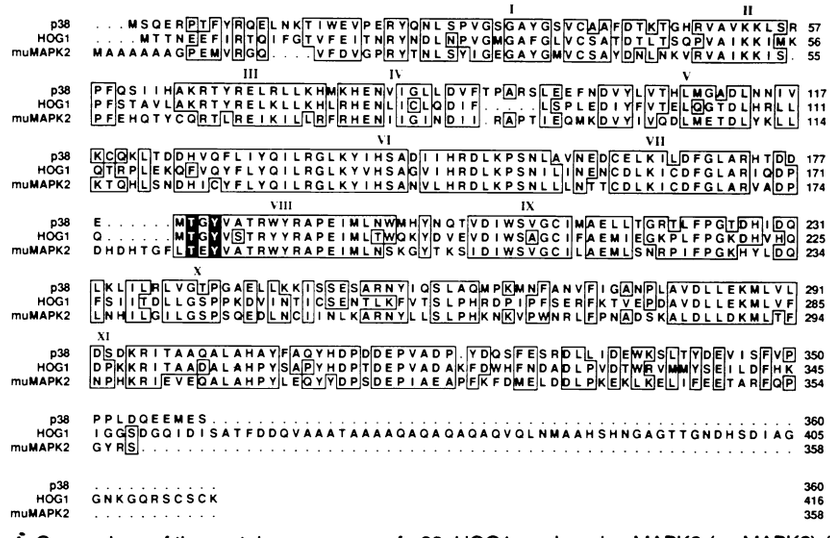

Fig. 1. Comparison of the protein sequences of p38, HOG1, and murine MAPK2 (muMAPK2) (*15*). Conserved subdomains that are present in all protein kinases are indicated by Roman numerals I to XI. Exact matches and conservative substitutions are within the boxed sequences. The phosphorylation sites are denoted by black boxes. Gaps (indicated by dots) were introduced to optimize the alignment (*12*).

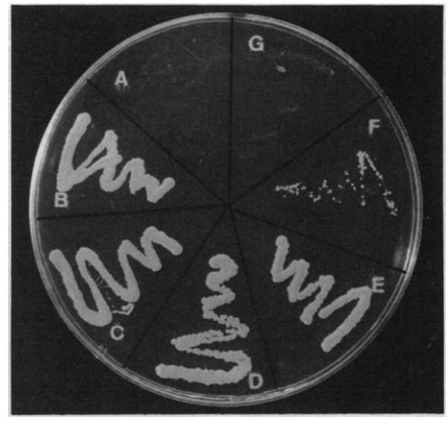

Fig. 2. Complementation of HOG1 mutant by p38 in *S. cervisiae*. The region of cDNA encoding p38 was subcloned in the yeast expression vector pAB23BXN (*18*). The orientation of the cDNA was determined by restriction digests. Yeast were transformed by electroporation with a setting of 2 mv, 25 μF, and 200 ohm. Transformants were selected and grown overnight in liquid Dex,Ura^- medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids, 0.0005% adenine, 0.0005% tryptophan, and 2% dextrose); the parental cells were grown in YEPD medium (1% yeast extract, 2% bactopeptone, and 2% dextrose); and equal numbers of cells were plated on a Dex agarose (Dex,Ura^- medium plus 0.0005% uracil and 2% agar) plate containing 0.9 M NaCl for testing growth. (**A**) HOG1 mutant strain JBY13 (*hog1-Δ1*). (**B**) Wild-type strain YPH102. (**C**) Strain YPH102 containing cDNA encoding p38 in pAB23BXN in wrong orientation. (**D**) Strain YPH102 containing cDNA encoding p38 in pAB23BXN in correct orientation. (**E**) Strain JBY13 containing pJB17 plasmid containing gene encoding HOG1 (*8*). (**F**) Strain JBY13 containing cDNA encoding p38 in pAB23BXN in correct orientation. (**G**) Strain JBY13 containing cDNA encoding p38 in pAB23BXN in wrong orientation.

M NaCl (Osm^S phenotype) but not as effectively as did a plasmid containing the yeast *HOG1* gene. A plasmid containing p38 in the opposite orientation failed to complement *hog1-Δ1*. Introduction of p38 slowed the growth of the transformants of several different yeast strains even in normal medium (*11*).

Similarities between p38 and HOG1 prompted us to investigate whether exposing mammalian cells to increased osmolarity would result in tyrosine phosphorylation of p38. Murine cell lines with MO characteristics (J774A.1 and RAW 264.7), as well as the murine pre–B cell line 70Z/3, transfected (*7*) with a vector (70Z/3-RSV) or with human CD14 (70Z/3-hCD14), were treated with hyperosmolar solutions of NaCl or sorbitol. These same cell lines were also treated with LPS or taxol. Taxol mimics LPS in murine cells and fails to activate MOs from mice unresponsive to LPS (*19, 20*). All of the cell lines contain MAPK1 and MAPK2 and p38 protein (*7*). Protein immunoblotting with an antibody to phosphotyrosine showed that p38 was tyrosine phosphorylated in all cell lines after exposure to hyperosmolarity or taxol (Fig. 3A). LPS induced tyrosine phosphorylation of p38 in all of the cell lines except the 70Z/3-RSV cells, which lack CD14 (*7*). LPS, taxol, and hyperosmolarity induced nearly identical patterns of protein tyrosine phosphorylation in the cell lines tested, which suggests the interrelatedness and stringency of the activation pathways initiated by these stimuli. Interleukin-1 also induced tyrosine phosphorylation of p38 but not of MAPK1 or MAPK2 in 70Z/3 cells (*11*). Hyperosmolar solutions induced phosphorylation of p38 within 5 min, and addition of as little as 0.1 M NaCl or 0.2 M sorbitol to the culture medium initiated this phosphorylation (*11*).

The MOs obtained from LPS-resistant mouse strains such as C3H/HeJ did not respond to either LPS or taxol; the basis of this genetic defect is unknown (*19, 21*). Because LPS, taxol, and hyperosmolar solutions all caused tyrosine phosphorylation of p38, we wondered if the effects of hyperosmolar solutions would be blunted in MOs obtained from peritoneal exudates (PEM) of C3H/HeJ(LPS^-) mice. Northern blot analysis showed that MOs derived from C3H/HeJ mice contained an amount of mRNA encoding p38 that was comparable to that found in other murine cell types (*11*). Phosphorylation of p38 was noted in PEM from C3HeB/FeJ(LPS^+) mice in response to hyperosmolarity, LPS, or taxol, whereas PEM from C3H/HeJ mice displayed increased p38 phosphorylation only in response to hyperosmolarity (Fig. 3B). Because CD14 expression in MOs from C3H/HeJ mice is normal (*11, 22*), these data suggest that the defect in C3H/HeJ mice that is responsible for hyporesponsiveness to LPS is localized to a functional component between the LPS receptor and p38. Identification of the upstream molecules that participate in p38 phosphorylation may provide new information about the genetic defect responsible for unresponsiveness to LPS.

MAPKs are a family of enzymes that regulate intracellular signaling pathways (*14, 23, 24*). Four isoforms of structurally related MAPKs have been identified in mammalian cells, and the existence of other MAPKs has been suggested (*25, 26*). In yeast and mammalian cells, several distinct pathways for MAPK activation have been identified that are highly conserved throughout evolution (*24*). One such pathway in yeast seems to be exclusively regulated by changes in extracellular osmolarity, and it results in tyrosine phosphorylation of HOG1 (*8*). Mammalian cells are often exposed to changing osmotic conditions. Selective ion channels probably participate in the processes that maintain and restore cell volume, but the underlying mechanisms that regulate these functions are not understood. Increased protein tyrosine phosphorylation associated with changes in osmolarity has been reported (*27*). We have found that at least a portion of the pathway in yeast that senses osmotic stress (*8*) appears to be conserved in mammalian cells. In yeast, HOG1 activation is associated with increased transcription of a group of genes that are termed stress genes (*28*). Lipopolysaccharide may be thought of as a stress signal to the cell because it alters normal cellular processes by inducing the release of mediators such as tumor necrosis factor that have systemic effects. It appears that both LPS and hyperosmolarity activate a signaling pathway that includes a common protein, p38. Identification of the upstream components of this pathway in mammalian cells may provide information about intracellular signaling pathways used by LPS and about signaling pathways that regulate mammalian cell responses to changing osmotic pressure.

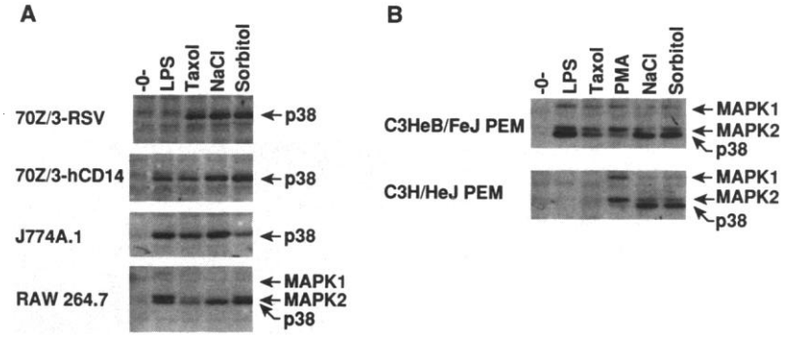

Fig. 3. Protein tyrosine phosphorylation induced by hyperosmolar solutions and other stimuli. Protein immunoblot analysis with antibody to phosphotyrosine was done as described (*7*). (**A**) Comparison of tyrosine phosphorylation of p38 in 70Z/3-RSV, 70Z/3-hCD14, J774A.1, and RAW 264.7 cells exposed to LPS (10 ng/ml), taxol (20 μM), 0.2 M NaCl, or 0.4 M sorbitol for 15 min. The position of p38 was verified by protein immunoblotting with antiserum to recombinant p38, and the positions of MAPK1 and MAPK2 were determined with antiserum to MAPK antiserum (*7*). (**B**) Effects of hyperosmolarity and other stimuli on protein tyrosine phosphorylation in MOs obtained from PEM of C3HeB/FeJ and C3H/HeJ mice (*7*). Additions and analyses of cell extracts were identical to those in (A). Cells were also treated with 0.1 μM phorbol myristate acetate (PMA). The effects of hyperosmolar solutions or of taxol do not result from contamination with LPS. All reagents were negative when tested with a chromogenic limulus assay. Antibodies to CD14 or to polymyxin B blocked the effects of LPS but did not inhibit phosphorylation induced by NaCl, sorbitol, or taxol. Heating the NaCl solution to 200°C for 72 hours (conditions that destroy LPS) failed to alter the cellular response to NaCl.

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10. The p38 was isolated from a lysate of 5×10^{10} 70Z/3-hCD14 cells treated with LPS (1 μg/ml) for 15 min as described (*7*); lysis was done in buffer A [20 mM tris-HCl (pH 7.5), 120 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride]. The cell-free lysate was applied to a 5-ml column containing an antibody to phosphotyrosine, conjugated to agarose (UBI, Lake Saranac, NY). After washing, the bound proteins were eluted with buffer A containing phosphotyrosine (1 mM). Eluted proteins were concentrated by acetone

precipitation, exchanged into 10 mM tris-HCl and 1 mM EDTA (pH 7.5) with a Bio-Rad p-10 column, and resolved by SDS–polyacrylamide gel electrophoresis (12% gel). The resolved proteins were electroblotted onto a polyvinylidene difluoride membrane; one lane of the sample (approximately 2% of the total protein) was immunoblotted with antibody to phosphotyrosine to localize p38, and the remainder of the blot was stained with Ponceau S. The stained p38 band was excised, and after treatment with trypsin (*29*), the resultant peptides were separated by high-performance liquid chromatography on a C18 column with a 0 to 100% acetonitrile gradient containing 0.01% trifluoroacetic acid (*29*). Fractions were collected, and a number of peptides were sequenced. All peptide sequences were found in the cDNA clone isolated. Degenerate oligonucleotides (16 mer) corresponding to either end of peptide 25, NFANVFIGANPLAVDLLEK (*15*), were used for the polymerase chain reaction (PCR) with cDNA from 70Z/3 cells as a template in order to obtain a DNA fragment containing the exact nucleotide sequences encoding the middle part of this peptide. Products of PCR resolved in an 8% acrylamide gel were eluted and subcloned into pBluescript vector. Sequencing verified the fidelity of the PCR product. A murine liver cDNA library (ClonTech, Palo Alto, CA) was screened with this PCR probe, and nine positive clones containing 0.8- to 3.5-kb inserts were obtained after screening 5×10^5 colonies. Restriction enzyme mapping and partial DNA sequencing indicated that all clones were identical; the longest cDNA clone was chosen for complete sequencing.

11. J. Han and R. J. Ulevitch, unpublished data.
12. The BLAST program was used to search the database through the National Center for Biotechnology Information. The PILEUP and PRETTYBOX programs were used for sequence alignments and comparisons. The FASTA program was used for calculating the identities of various protein kinases and of p38. The calculation of percent identity was done with 360 amino acids. All computer programs were from the Wisconsin Genetics Computer Group, Madison, WI.
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TECHNICAL COMMENTS

Biological Control and Refuge Theory

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In classical biological control, biologists introduce an imported parasitoid in order to reduce the density of an accidentally imported insect host. If the pest density declines and the introduced natural enemy achieves moderate-to-high frequencies of attack, projects are considered successful. An association between successful biological control and high parasitization is to be expected. Hawkins *et al.* (*1*) found that maximum parasitization rates occurred when hosts had little protection from parasitoids (that is, small refuges), and they conclude (*1*, p. 1430) that susceptibility to parasitism (that is, high rates of parasitism) is a "significant estimate of the probability that the parasitoid introduction will reduce host densities." We find this conclusion suspect for five reasons.

1) The literature used by Hawkins *et al.* is likely to be biased in favor of the refuge hypothesis because cases of pest populations declining with low rates of parasitization are unlikely to be recorded as examples of successful biological control (*2*).

2) Refuge size is estimated by Hawkins *et al.* as one minus the proportion of the pest population parasitized. By definition, high parasitism characterizes successful biological control, therefore the argument that small refuges favor the success of biological control is a tautology.

3) Most simple measurements of "percent parasitism" contain errors or distortions arising from the influences of host and parasitoid phenologies (*3*). Single values cannot characterize host-parasitoid interactions, and maximum values are unlikely to be typical over longer times or in different areas (*4*).

4) Contrary to the prediction made by Hawkins *et al.*, successful biological control can result from the use of agents that are characterized by low rates of parasitization in their native habitat (*5*). Natural enemies that are rare in their native habitat may have superior potential as control agents when released in exotic habitats (*6*).

5) Hawkins *et al.* attribute seven cases of high parasitization (above 60%) in unsuccessful biological control projects to climatic mismatch between parasitoids and hosts. We question how such high rates of parasitization could be achieved if climatic factors "reduce parasitoid reproduction, survivorship, or host synchrony ..." (*1*, p. 1431).

Variation in the susceptibility of insects to predators, parasitoids, and disease is important. Mechanisms for encapsulating parasitoids, hiding from predators, and resisting disease influence the impacts of natural enemies in native and exotic habitats, but measuring refuge size from the observed maximum parasitization of successful biological control programs does not yield new understanding or predictability to the practice of biological control.

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Hawkins *et al.* (*1*) demonstrate a significant and robust relationship between the outcome (success or failure) of a potential biocontrol program and the maximum percentage parasitism achieved by the parasitoid agent following its initial release. They propose that this relationship illustrates that the size of a "ref-