even among antigen-binding cells after hyperimmunization, let alone in B cells from unimmunized animals. Even if the unstudied 80% idiotype-positive cells were devoid of mutations, the overall frequency would still be close to 1%.

With a standard enzyme-linked immunosorbent assay (ELISA), we determined the concentrations of various Ig isotypes in the sera of QM and control mice (Table 1). As expected, B cells in the QM mouse can and do switch from IgM to other isotypes. The data in Table 1 also provide clues to the origin of serum Ig in unimmunized animals, one of the oldest unsolved problems in immunology. If network interactions among surface receptors (*17*) were necessary to produce serum Ig, its concentration should be low in QM mice. However, QM mice tended to have higher concentrations of the various isotypes, especially IgG2a and IgA, than did control animals housed under the same conditions. QM mice 5 and 6 were mildly and highly hypergammaglobulinemic, respectively. Less than half the IgM and none of the other isotypes tested bound NP, confirming the flow cytometric data showing that monospecificity evolves into diversity in these mice. Cells with mutant receptors appear to be preferentially expanded and switched. We can also exclude a simple stochastic model in which serum Ig would be a consequence of random activation of a small fraction of B cells. Such a model would predict that the fraction of serum Ig that binds NP would reflect the fraction of B lymphocytes that bind NP. Although the B cell content of the entire mouse was not surveyed, most B lymphocytes in peripheral blood and spleen bind NP, and thus the stochastic model would predict that most of the serum Ig would bind NP. In summary, we can rule out a purely network model and a purely random stochastic model for the origin of serum Ig. Although serum Ig represents a record of previous antigenic exposure, some type of homeostatic control must also play a role.

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8. Approximately 5.6% of the cells express λ1 and 2.4% express λ2, yielding a λ1/λ2 ratio of 70/30, as determined by PE-coupled anti-μ^a and FITC-coupled anti-λ1 and anti-λ2. In a C57BL/6 control mouse, the λ1/λ2 ratio was 76/24. Because the binding site of the monoclonal anti-λ1 is not known, λ3 is detected together with either λ1 or λ2.
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12. Material precipitated by sequential incubation of cells with NP conjugated to BSA and antibodies to BSA contained the same polypeptide chains—μ, λ1, and λ2—as material precipitated with an antiserum to μ and λ. No such material was precipitated with NP-BSA from cells of a C57BL/6 mouse nor with BSA lacking the hapten NP from cells of QM or C57BL/6 mice. These results confirm our flow cytometric data showing that most B cell receptors in the QM mouse bind antigen.
13. Only the 3′ end with the embedded heptamer of each V_H is shown in Fig. 4. Except for a few base pair differences, the new V_H segments correspond with known V_H sequences in the Kabat *et al.* [E. A. Kabat, T. T. Wu, H. M. Perry, K. S. Gottesman, C. Foeller, *Sequences of Proteins of Immunological Interest* (NIH, Bethesda, MD, ed. 5, 1991)] and GenBank databases. The lack of identity is likely attributable to hypermutation and the fact that the new V_H segments are derived from mouse strain 129, whereas those in the databases are derived from other strains, principally BALB/c. These details are currently being resolved; in the present context, it is important only that they are not similar to V_H17.2.25.
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18. The 16.3-kb plasmid t-v.1 contains a rearranged μ_H chain gene, without the membrane exons, and the selectable markers *neo*^{r} and *tk*, which are both in an orientation opposite that of the inserted anti-NP μ gene. The 17.2.25 V region contains V_H10 joined to $D_{SP2.3}$ and J_H4; it is flanked by DNA sequences homologous to those 5′ and 3′ of the stretch containing the J_H segments in the normal heavy chain locus. The 3′ sequence contains the major intron and C_μ. The shorter, 5′ sequence contains DQ52, the most proximal D segment, and flanking sequences. The cloning strategy was as follows: The 5′ homologous region, a 1.6-kb Hind III–Bam HI DNA fragment containing DQ52, was cloned into the same sites of Pmc1Neo (Stratagene). Subsequently, the Xho I site 5′ of the DQ52 segment was destroyed by nucleotide incorporation with the Klenow fragment and blunt-end ligation. The Xho I–Sal I herpes simplex virus–thymidine kinase (*HSVtk*) gene was then cloned into the remaining Xho I site. The Hind III site 5′ of DQ52 was also eliminated to allow cloning of the heavy chain gene [the Sal I–Xho I fragment from plasmid pμ (*3*)] into the Hind III site 5′ of the *HSVtk* gene.

19. We thank L. Reichardt and G. Martin for help with producing the QM mouse; R. Grosschedl for the plasmid pμ; T. Imanishi-Kari for providing antibodies to V_H17.2.25 and NP-BSA; D. Huszar and N. Lonberg of GenPharm for providing the heavy chain and κ double knockout mice; R. Murray and P. Vieira for advice on generating replacement mice; and C. Steinberg for editorial assistance. Supported by NIH grants 1R01 GM37699 and P60 AR20684, the Engalitcheff Award of the Arthritis Foundation, the Lucille P. Markey Charitable Trust, the Junta Nacional de Investigação Científica e Tecnológica–Praxis XXI-BD 3763/94 (M.C.), and the American-Italian Foundation for Cancer Research (L.M.).

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Identification of MAP Kinase Domains by Redirecting Stress Signals into Growth Factor Responses

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Mitogen-activated protein kinase (MAPK) cascades, termed MAPK modules, channel extracellular signals into specific cellular responses. Chimeric molecules were constructed between p38 and p44 MAPKs, which transduce stress and growth factor signals, respectively. A discrete region of 40 residues located in the amino-terminal p38MAPK lobe directed the specificity of response to extracellular signals, whereas the carboxyl-terminal half of the molecule specified substrate recognition. One p38-p44MAPK chimera, expressed in vivo, redirected stress signals into early mitogenic responses, demonstrating the functional independence of these domains.

MAP kinases form a large family of serine-threonine protein kinases activated by separate cascades conserved through evolution (*1*). In mammalian cells, three distinct MAPK cascades have been identified: p42-p44 MAPKs (*2*), p38 MAPK (*3*, *4*), and p46-p54 JNKs (*5*). Activation of p42-p44 MAPKs constitutes a crucial step in the pathway mediating cell proliferation in re-

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sponse to growth factors (6), whereas p38MAPK and JNK mediate signals in response to cytokines and environmental stress (3–5). The fact that these biological responses are distinct implies that the specificity of external stimuli has to be maintained throughout each of the cascades. This specificity can result from the selective enzyme-substrate interactions within a particular module. For instance, each MAPK is specifically phosphorylated and activated in vitro by an upstream kinase (MKK1 or 2 for p42-p44MAPKs, MKK3 for p38MAPK, and MKK4 for JNK) (*4*, *7*), implying that specific constraints must have evolved to prevent cross-activation.

The selectivity of activation by external stimuli is illustrated by an in vitro assay of hemagglutinin (HA)–tagged forms of p38 and p44 MAPKs (Fig. 1A). When expressed in 293 cells, p44MAPK was preferentially activated by growth factors [serum, epidermal growth factor (EGF) plus insulin], whereas p38MAPK was selectively stimulated by stress [sorbitol or anisomycin in 293 cells (Fig. 1A) and interleukin-1β (IL-1β) in CCL39 hamster fibroblasts (*8*)]. The p38 and p44 MAPKs also exhibit a distinct substrate selectivity: Whereas myelin basic protein (MBP) and the transcription factor Elk1 were phosphorylated to the same extent by both MAPKs, p44MAPK preferentially phosphorylated Myc, and p38MAPK preferred ATF2 as a substrate (Fig. 1B).

Hence, despite the high degree of similarity (60%) between p38 and p44 MAPKs (Fig. 1C), their activation and substrate recognition occur in a specific manner in vitro. To define the domains responsible for this specificity, we generated chimeric molecules between both MAPKs. We first investigated the role of the T-loop in the specificity of activation, because this DFG to APE variable region (*9*) contains the key threonine and tyrosine residues responsible for MAP kinase activation upon phosphorylation by the appropriate MKK (Fig. 1C). To this end, based on the three-dimensional (3D) model of protein kinases (*10*), we fused the NH_2-terminal lobe of p38MAPK to the COOH-terminal lobe of p44MAPK (Fig. 2A). The fusion site was made either in kinase subdomain VIII (*11*), so that the T-loop came from p38MAPK (p38-p44 VIII), or in the subdomain VII, so that the T-loop came from p44MAPK (p38-p44 VII). Surprisingly, both chimerae were stimulated only by stress signals, irrespective of the origin of the T-loop (Fig. 2B), suggesting that this region is not crucial for the specificity of activation. A p38MAPK containing only the p44MAPK T-loop [p38-(p44 T-loop)-p38] was completely inactive (Fig. 2D), indicating that the integrity of the T-loop may be essential for kinase activity. However, this chimera displayed a reduced electrophoretic mobility in response to stress agonists exclusively (Fig. 2D) and is therefore likely to be phosphorylated by the kinase that activates p38MAPK (MKK3), despite the fact that its T-loop was that of p44MAPK. These results indicate that the domain conferring specificity of stress activation is not the T-loop by itself but is located NH_2-terminal to that region.

To map more precisely the p38MAPK activation domain, we progressively replaced the NH_2-terminal region of p38MAPK with the corresponding domains of p44MAPK (Fig. 2A). The chimera in which the fusion site was made in domain V (p38-p44 V) was still strongly activated by stress agonists, although it acquired partial responsiveness to growth factors (Fig. 2B). By contrast, the chimeras in which the fusion site was made in subdomain III (p38-p44 III) or in domain I (p38-p44 I) were activated by growth factors rather than stress (Fig. 2B). These findings suggest that a critical region dictating the specificity of activation is localized between kinase subdomains III and V, which corresponds to the αC helix, the L5 loop, the β4 sheet, the L6 loop, and the beginning of the β5 sheet (Fig. 1C), according to the 3D model of p42MAPK (*12*).

To define the MAPK region involved in the specificity of substrate recognition, we assayed the ability of the chimeras to phosphorylate ATF2 or Myc, preferential substrates of p38 and p44MAPKs, respectively. The p38-p44 VIII and VII chimeras phosphorylated ATF2 and Myc to the same extent (Fig. 2C), indicating that substrate recognition involved both the NH_2- and COOH-terminal lobes, which correlates with predictions made from the 3D structure of p42MAPK (*12*). More precisely, because the p38-p44V chimera switched toward a p44MAPK-like substrate specificity, the substrate recognition area appears to be restricted to a region COOH-terminal of the domain V (Fig. 2C). Hence, the specificity of substrate recognition involves a large domain, localized mainly in the COOH-terminal region of the kinase.

The dissociation of domains conferring specificity of activation and substrate recognition allowed us to obtain a chimera (p38-p44 V) that was activated by stress, like p38MAPK, but that displayed a p44MAPK specificity of substrate recognition in vitro (Fig. 2, A to C). An important implication of this finding is that if this chimera behaved similarly in vivo, its response to stress agents should be redirected into mitogenic events. We thus investigated three early growth factor responses known to be under the control of the p42-p44MAPK cascade: (i) activation of the protein kinase $p90^{rsk}$ (*4*, *13*); (ii) activation of the c-*fos* promoter (*14*); and (iii) activation of the *cyclin D1* promoter (*15*). As shown in Fig. 3A (upper panel), $p90^{rsk}$ coimmunoprecipitated with p44MAPK and the

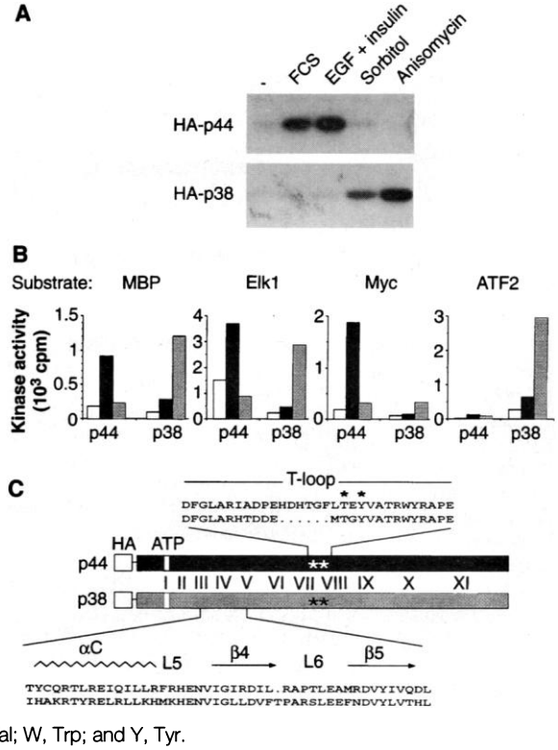

Fig. 1. Activation, substrate recognition, and sequence comparisons between p38 and p44 MAPKs (*19*). (**A**) Activation by external stimuli. Several agonists were assayed: fetal calf serum (FCS) (20%, 5 min), EGF (50 ng/ml) plus insulin (5 μg/ml, 15 min), sorbitol (300 mM, 15 min), or anisomycin (50 ng/ml, 20 min). Times of stimulation (in parentheses) correspond to the optimal stimulation times. The substrates used were the specific substrates for each kinase as defined (Fig. 1B). (**B**) Substrate selectivity. The p44 and p38 MAPKs were activated by FCS (solid bars) or anisomycin (shaded bars) and assayed with various substrates (MBP, Elk1, Myc, and ATF2). (**C**) Comparison between mouse p38 (*3*) and Chinese hamster p44 (*18*) MAPK protein sequences. Kinase subdomains (*11*) are indicated, and the phosphorylation sites are marked by asterisks. The ATP-binding site is shown by a white box. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; 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.

p38-p44 V chimera but not with p38MAPK, confirming that also in vivo the chimera has a p44MAPK-like specificity of substrate. However, $p90^{rsk}$, which was phosphorylated in response to growth factors when associated with p44MAPK, became phosphorylated in response to anisomycin when associated with the p38-p44 V chimera (Fig. 3A, middle panel). Moreover, with S6 as a substrate, $p90^{rsk}$ was preferentially activated in response to growth factors (serum) and not by stress (anisomycin) in control cells (Fig. 3B). This pattern of activation was not modified by the ectopic expression of p44 or p38 MAPKs. By contrast, in cells expressing the p38-p44 V chimera, $p90^{rsk}$ could be activated by anisomycin. [In cells expressing the chimera, activation by serum is due to endogenous p42-p44 MAPK activation (see control).] This result demonstrates that in vivo, the p38-p44 V chimera associates with, phosphorylates, and reactivates a p44 MAPK substrate in response to stress.

Furthermore, in CCL39 fibroblasts, the c-*fos* promoter is activated in response to growth factors (serum), as a consequence of activation by the endogenous p42-p44 MAPKs, but not by stress (IL-1β), indicating that endogenous p38MAPK has no role in the activation of this promoter (Fig. 3C). The ectopic expression of p44 or p38 MAPKs slightly modified the activity of the promoter but did not change its pattern of activation by external stimuli. By contrast, the expression of the p38-p44 V chimera results in the activation of this promoter by IL-1β. As noted for $p90^{rsk}$, serum activation of the c-*fos* promoter represents the contribution of the endogenous p42-p44 MAPKs. We obtained similar results with the *cyclin D1* promoter (*16*). Thus, the p38-p44 V chimera is able to redirect stress signals into early growth factor responses. These in vivo results validate the identification of the MAPK domains directing specificity of activation and substrate recognition obtained in vitro.

We have identified and mapped a region directing specificity for stress activation in the p38MAPK NH_2-terminal lobe. Interestingly, this region contains an exposed αC helix that, because of its proximity to the catalytic cleft, is a good candidate for being

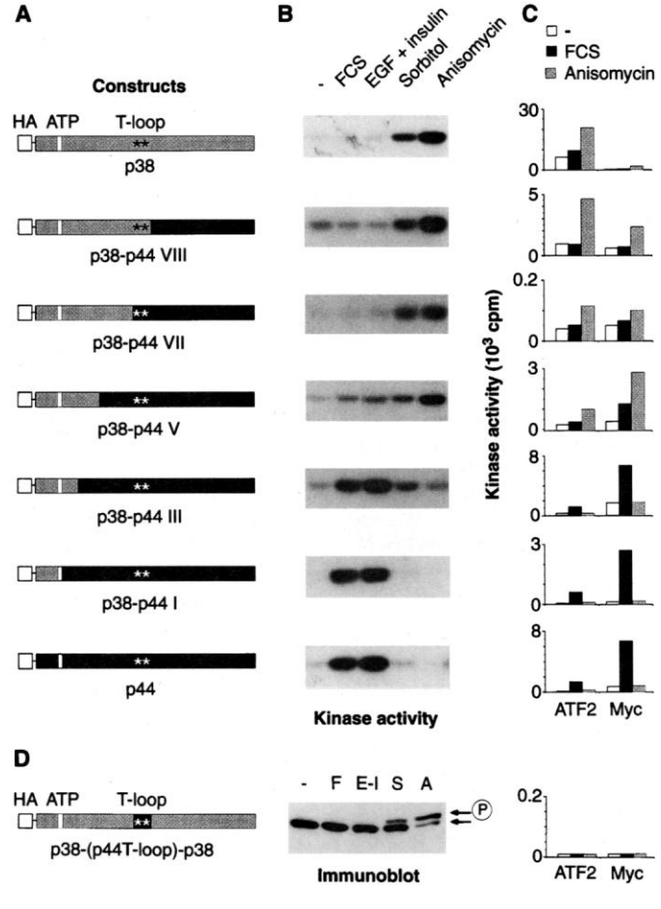

Fig. 2. Activation and substrate recognition of chimeric molecules between p38 and p44 MAPKs. (**A**) Constructs. Chimeric molecules were constructed between HA-tagged p38 and p44 MAPKs (*20*). Phosphorylation sites are represented by asterisks. The ATP binding site is shown by a white box. The chimeras were transfected into 293 cells and their activity was determined as described (Fig. 1) (*19*). (**B**) Activation of the chimeras by external stimuli. Several agonists were assayed as described (Fig. 1A). The substrates used were those defined as being the best substrate for each kinase (Fig. 2C). (**C**) Substrate selectivity of the chimeras. The chimeras were stimulated by FCS (20%, 5 min) (solid bars) or anisomycin (50 ng/ml, 20 min) (shaded bars) and assayed with ATF2 or Myc as substrates. Identical results were obtained in three independent experiments. In general, chimeric molecules are less active than the parental p38 and p44MAPKs. To simplify comparison, the scale of the *y* axis is maximal for each chimera, allowing an evaluation of the ratios for agonist activation and substrate specificity. (**D**) The fragment corresponding to the p38MAPK T-loop (DFG to APE sequence) was replaced by that of p44MAPK. The construct was transfected into 293 cells, and cells were stimulated by various agonists as described (Fig. 1A). F, FCS; E-I, EGF plus insulin; S, sorbitol; and A, anisomycin. Lysates were analyzed by immunoblotting with anti-HA (*21*). The upper band represents the phosphorylated form of this chimera.

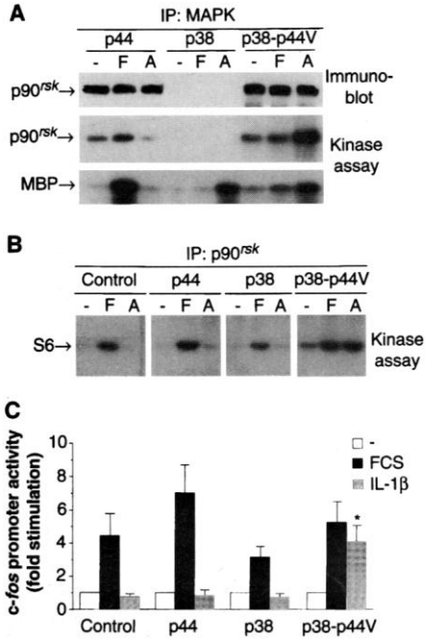

Fig. 3. Effect of the stress-activated p38-p44 V chimera on p44MAPK-dependent events. (**A**) $p90^{rsk}$ association and phosphorylation by MAPKs. The constructs encoding the wild-type (WT) p44 and p38 MAPKs or the p38-p44 V chimera were transfected with the plasmid encoding avian $p90^{rsk}$ into 293 cells. Cells were either not stimulated (−) or stimulated with FCS (20%, 5 min) or anisomycin (50 ng/ml, 20 min). MAPKs were immunoprecipitated with anti-HA, and the immunoprecipitates were either resolved on SDS-PAGE and immunoblotted with anti-$p90^{rsk}$ (upper panel), subjected to a kinase assay without the addition of any substrate (to magnify associated $p90^{rsk}$ phosphorylation) (middle panel) or subjected to a kinase assay with MBP as a substrate (to verify MAPK activity) (*22*). (**B**) $p90^{rsk}$ activity. The vector pECE alone or the constructs encoding the corresponding MAP kinases: WT p44 and p38 MAPKs or the p38-p44 V chimera were transfected with the plasmid encoding the avian $p90^{rsk}$ into 293 cells. Cells were either not stimulated (−) or stimulated with FCS (20%, 5 min) or anisomycin (50 ng/ml 20 min). The transfected $p90^{rsk}$ was selectively immunoprecipitated with avian anti-$p90^{rsk}$ and assayed with 40*S* ribosomal protein S6 as a substrate (*23*). Identical results were obtained in three independent experiments. (**C**) c-*fos* promoter activity. The vector pECE alone or the constructs encoding WT p44 and p38 MAPKs or the p38-p44 V chimera were transfected with the reporter gene luciferase under the control of the c-*fos* promoter into CCL39 fibroblasts. Cells were serum-deprived for 24 hours and stimulated for 16 hours with FCS (20%) (solid bars) or IL-1β (10 ng/ml) (shaded bars). Luciferase activity was then determined in cell lysates (*24*). Values shown are the mean ± SEM of five independent experiments conducted in triplicate. (*) Significantly different from the other IL-1β values ($P < 0.02$, Student's *t* test).

contacted by the appropriate upstream kinase (MKK3 in this instance) and directing it to phosphorylate the residues in the T-loop. Indeed, the αC helix of Cdk2 interacts with the regulatory subunit cyclin A, reorienting the T-loop and opening the entrance of the catalytic cleft (*17*). The αC helix might therefore represent a key exposed region used by protein kinases to receive input regulatory signals.

By generating chimeric MAPK able to convert stress signals into growth factor responses, we have demonstrated that signal reception domains may be dissociable from signal delivery domains. If this model can be generalized to other members of the MAP kinase family as well as to the upstream kinases of the transduction cascades, a new class of specific "MAPK module" antagonists could be created by targeting domains specifying agonist activation.

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19. p44MAPK was NH_2-terminally tagged with the HA epitope (MYDVPDYASLP) replacing the first 10 codons and subcloned in the pECE expression vector driven by an SV40 promoter. Murine p38MAPK complementary DNA (cDNA) was NH_2-terminally tagged at the first codon with the HA epitope and subcloned in the pECE vector. The 293 cells (6×10^5 cells per 35-mm well) were transfected with 6 μg of DNA by the calcium phosphate technique. Two days after transfection, cells were serum-deprived for 5 hours and stimulated with various agonists. Kinase assays were done as described (*18*). Briefly, cells were lysed in 400 μl of Triton lysis buffer. Equal amounts of proteins (300 μg) were immunoprecipitated on protein A–Sepharose beads coupled with the antibody to HA (anti-HA) (Babco, Emeryville). Activity of the kinases were assayed in 40 μl of kinase buffer with various substrates: glutathione-S-transferase (GST)–ATF2 (1–109), GST-Myc (1–143), GST-Elk1 (307–428), and MBP at a final concentration of 10 μg and 3 μCi of [γ-^{32}P], 50 μM adenosine 5′-triphosphate (ATP) (ICN) for 30 min at 30°C. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS–polyacrylamide gel electrophoresis (PAGE); the gels were autoradiographed, stained with Coomassie brilliant blue, and the bands corresponding to the substrates were excised and counted.
20. All chimerae were constructed from p44 and p38 MAPK vectors by introduction of polymerase chain reaction (PCR) fragments through use of restriction sites in p38MAPK [Ava I [93 base pairs (bp), domain I], PpuM I (200 bp, domain III), BstE II (312 bp, domain V), Sac I (491 bp, domain VII), and Kpn I (564 bp, domain VIII)] or in p44MAPK [Afl II (508 bp, domain VII) and Kpn I (600 bp, domain VIII)]. All the PCR fragments and the junctions were verified by sequencing.
21. Protein immunoblot experiments were done with anti-HA after resolution of proteins on SDS-PAGE (*18*).
22. The 293 cells (6×10^5 cells per 35-mm well) were cotransfected with 0.2 μg of the plasmid encoding avian p90^{rsk} and 1 μg of the relevant constructs. Two days after transfection, cells were serum-deprived for 5 hours and stimulated by various agonists. Cells were lysed in the Triton lysis buffer, and equal amounts of proteins (300 μg) were immunoprecipitated with anti-HA. Kinase assays were done for 30 min at 30°C either with 10 μg of MBP, 3 μCi of [γ-^{32}P], 50 μM ATP to measure MAPK activity or with 3 μCi of [γ-^{32}P], 1.5 μM ATP to measure phosphorylation of associated p90^{rsk}. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS-PAGE (7.5%); the gel was autoradiographed, transferred onto nitrocellulose, and immunoblotted with an antibody directed against p90^{rsk} (Santa Cruz).
23. The 293 cells (6×10^5 cells per 35-mm well) were cotransfected with 3 μg of the plasmid encoding avian p90^{rsk} and 3 μg of the relevant constructs. Two days after transfection, cells were serum-deprived for 5 hours and stimulated by various agonists. Cells were lysed in the Triton lysis buffer, and equal amounts of proteins (300 μg) were immunoprecipitated with an antibody directed against avian p90^{rsk}. Kinase assays were done as described [R.-H. Chen and J. Blenis, *Mol. Cell. Biol.* **10**, 3204 (1990)] in 40 μl of kinase buffer, 20 μg of 40S subunit, and 3 μCi of [γ-^{32}P], 50 μM ATP for 15 min at 30°C. Reactions were stopped by addition of 25 μl of Laemmli buffer and heated for 5 min at 95°C. Samples were resolved on SDS-PAGE and the gel was autoradiographed.
24. CCL39 cells (10^5 cells per well in 24-well culture plates) were transfected with 0.25 μg of the c-*fos*–luciferase reporter gene and 0.75 μg of the relevant constructs. One day after transfection, cells were incubated in serum-free medium for 24 hours, then stimulated with fetal calf serum (20%) or IL-1β (10 ng/ml; Boehringer) for 16 hours. Luciferase activity was measured according to the Promega protocol.

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Enzymatic Synthesis of a Quorum-Sensing Autoinducer Through Use of Defined Substrates

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Many bacteria, including several pathogens of plants and humans, use a pheromone called an autoinducer to regulate gene expression in a cell density–dependent manner. *Agrobacterium* autoinducer [AAI, *N*-(3-oxo-octanoyl)-L-homoserine lactone] of *A. tumefaciens* is synthesized by the TraI protein, which is encoded by the tumor-inducing plasmid. Purified hexahistidinyl-TraI (H_6-TraI) used *S*-adenosylmethionine to make the homoserine lactone moiety of AAI, but did not use related compounds. H_6-TraI used 3-oxo-octanoyl–acyl carrier protein to make the 3-oxo-octanoyl moiety of AAI, but did not use 3-oxo-octanoyl–coenzyme A. These results demonstrate the enzymatic synthesis of an autoinducer through the use of purified substrates.

Mechanisms that allow bacteria to control gene expression in a cell density–dependent manner have evolved independently a number of times (*1*, *2*). This type of gene expression is referred to as quorum-sensing. Probably the best characterized quorum-sensing system is the LuxR-LuxI system of *Vibrio fischeri*. LuxI protein produces *V. fischeri* autoinducer [VAI, *N*-(3-oxo-hexanoyl)-L-homoserine lactone], which binds to the transcriptional activator protein LuxR (*3*, *4*). Complexes of LuxR-VAI activate transcription of the *lux* operon, resulting in bioluminescence. Because VAI diffuses passively across the cell envelope, high intracellular concentrations of VAI are attained only in the presence of neighboring VAI-producing bacteria (*5*). Similar regulatory systems are found in a broad variety of eubacteria, many

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