JAK2, Ras, and Raf Are Required for Activation of Extracellular Signal-regulated Kinase/Mitogen-activated Protein Kinase by Growth Hormone*  

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Protein-tyrosine kinases (PTKs) of the JAK family have been characterized on the basis of their ability to mediate the rapid induction of transcription of interferon-responsive genes through the stimulation of a class of latent cytoplasmic transcription factors known as signal transducers and activators of transcription (STATs). STAT activation, which has been described as being Ras-independent, requires tyrosine phosphorylation, but STAT transactivating activity is enhanced by phosphorylation on serine as well, probably by extracellular signal-regulated kinase/mitogen-activated protein kinase(s) (ERK/MAPK). STATs can be activated upon binding of ligands to receptor PTKs, to G-protein-linked receptors, and to cytokine receptors. Whether JAKs are required for the activation of signaling pathways other than that leading to STAT activation is not known. The binding of growth hormone (GH) to its receptor (GHR) activates JAK2 and STATs as well as ERK/MAP kinases. We have used a transient transfection system in 293 cells to evaluate the requirement for JAK2 in the activation of ERK2/MAPK by GH. We found that JAK2 is required for GH-stimulated activation of ERK2/MAPK. Employing the transient expression of dominant negative forms of H-Ras and Raf-1, we determined that the GHR/JAK2-mediated activation of ERK2/MAPK is dependent on both Ras and Raf. Thus, JAK protein-tyrosine kinases may represent a common component in the activation of the ERK2/MAPK and STAT signaling pathways, which appear to bifurcate upstream of Ras activation but converge with ERK/MAPK phosphorylation of STATs.

A role for the Janus kinase (JAK) family of protein-tyrosine kinases in cell signaling was first defined by the observation that the expression of receptor-specific JAK proteins is required for the rapid induction of interferon (IFN)-responsive genes. This activity is mediated by the activation of STAT proteins that promote transcription via binding to interferon-specific response elements (1). In addition to mediating transcription of interferon-responsive genes, JAK/STAT signaling pathways (2) are now thought to mediate similar responses to angiotensin II, which binds to a G-protein-coupled receptor (3), and to several cytokines, including growth hormone (GH), prolactin, erythropoietin (EPO), leukemia inhibitory factor, ciliary neurotrophic factor, granulocyte-CSF and granulocyte-macrophage-CSF, oncostatin M, and several interleukins (4, 5). The persistence of the IL-6-induced DNA binding activity of STAT3 (6) and the PDGF-stimulated DNA binding activity of STAT1α (7) in nuclear extracts of cells expressing dominant negative Ras suggests that at least some aspects of STAT activation are Ras-independent.

The binding of GH to its receptor (GHR) induces receptor homodimerization (8, 9) and stimulates the tyrosine phosphorylation of JAK2 (10) and the DNA binding activity of STAT1α (11), 3 (12), and 5 (13). Like other cytokines, GH can also activate ERK/MAP kinases (MAPK) (14–16). The activation of the ERK/MAPK pathway, which in most systems requires Ras and Raf, is a universal downstream response to the activation of most receptor PTKs. It is frequently the case that ligand-stimulated receptor autophosphorylation results in the creation of receptor phosphotyrosine docking sites for the binding of the SH2 domain-containing proteins Grb2 or Shc, which can bind Grb2, bound to effectors of Ras activation. By analogy, then, it has been presumed that cytokine activation of the ERK2/MAPK pathway would first require the recruitment by ligand activated cytokine receptors of one or more PTKs in order to mimic, at least qualitatively, this role of receptor PTKs.

The maximal activation of STAT1α and STAT3-mediated transcription requires phosphorylation on both tyrosine and serine residues (2). That JAK/STAT pathways might be regulated in part by ERK2/MAPK is suggested by the observations that the activating serine phosphorylation site in STATs 1 and 3 is in an ERK2/MAPK phosphorylation consensus sequence, that IFNβ-activated STAT1α communoprecipitates with ERK2/MAPK, and that the kinase activity of ERK/MAPK is required for IFNβ-stimulated gene transcription (18). However, the issue of whether JAKs are involved in the regulation of the ERK2/MAPK pathway remains unresolved (19). To test the possibility that JAK proteins might be required for the activation of the ERK2/MAPK pathway by GH, we transiently expressed the GHR with or without JAK1, JAK2, or a kinase-inactive form of JAK2 in E1A-immortalized human embryonic kidney (293) cells, and the effect on the activity of an epitope-tagged form of ERK2/MAPK was evaluated. Our results indicate...

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1 The abbreviations used are: JAK, Janus kinase; STAT, signal transducers and activators of transcription; ERK/MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; CSF, colony-stimulating factor; MEK, MAP kinase kinase; MEKK, MAP kinase kinase kinase; IRS-1, insulin receptor substrate 1; IL, interleukin; GH, growth hormone; GHR, growth hormone receptor; EPO, erythropoietin; EPOR, erythropoietin receptor; PTK, protein-tyrosine kinase; IFN, interferon; HA, hemagglutinin; RIPA, radiommune precipitation assay buffer; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; TPA, 12-O-tetradecanoylphorbol-13-acetate; SRE, serum response element; SIE, sis-inducible factor response element.
cate that JAK2 is required for the activation of ERK2/MAPK by GH. We evaluated the involvement of Ras and Raf in JAK2-mediated activation of ERK2/MAPK by GH by transiently coexpressing dominant negative forms of either H-Ras or Raf-1 with the GHR and JAK2 and found that the GHR/JAK2-mediated activation of ERK2/MAPK was inhibited both by dominant negative Ras and dominant negative Raf.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Reagents—Human embryonic kidney (293) cells were maintained in DMEM containing 10% FBS. Human pituitary growth hormone, which was used at 20 nM in all experiments, was generously provided by Dr. Jack Kastly. A monoclonal antibody (12CA5) to the hemagglutinin epitope tag of ERK2/MAPK (20) was used both for immunoprecipitation and for immunoblotting of HA-ERK2/MAPK for in vitro kinase assays. The expression of the transfected GHR was confirmed by immunoblotting with a rabbit polyclonal antibody to the cytoplasmic domain of the GHR fused to GST. The increase in JAK2 levels following transfection was evaluated by immunoprecipitation/imunoblotting with a polyclonal antibody to a C-terminal peptide of JAK2 (Santa Cruz).

Expression Vectors and Transfection—The full-length murine GHR cDNA, provided in a pLNCAL7 vector (20), was subcloned into a pSXL-CMV mammalian expression vector. The full-length wild-type murine JAK2 and JAK1 cDNAs, provided by Dr. Rikiko Fukunaga, or a kinase-inactive form of the murine JAK2 cDNA, provided by Dr. Don Wojchowski (22), had been subcloned into pEF-BoS expression vectors (23). The HA-tagged p42 ERK2/MAPK cDNA, provided by Dr. Michael Weber, was in a pLNCAL7 vector (20). The dominant negative (S17N) H-Ras cDNA in pSRα, the dominant negative (K375W) Raf-1 and wild-type Raf-1 cDNAs in pRSV, and the cDNA for the constitutively active MEKK1 in pCMV5 (24) were provided by Dr. Michael Karin. For experiments, cells were cultured in 6-cm dishes precoated with poly-L-lysine and, when subconfluent, were transfected with Lipofectamine, as per the manufacturer's instructions (Life Technologies, Inc.), and harvested 36 h later following 16 h of incubation in serum-free medium.

Immunoblotting, Immunoprecipitation, and in Vitro Kinase Assays—For immunoblotting, cells were solubilized in ice-cold RIPA (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM sodium vanadate, 50 μM leupeptin, 1 μM phenylmethylsulfonyl fluoride, and 10% aprotinin). Lysates were clarified by centrifugation at ~25,000 g at 4°C, and supernatants were assayed for protein levels (Bio-Rad) for normalization of protein contents. Washed beads were incubated for 10 min at 30°C in 20 μl of kinase reaction buffer (20 mM Hapes, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol). Washed beads were incubated for 10 min at 30°C in 20 μl of kinase reaction buffer containing 20 μM ATP, 10 μCi of 32P-ATP (3000 Ci/mmol; Amersham), and 10 μg of myelin basic protein (Sigma). Reactions were terminated by the addition of 2 x sample buffer and boiling. Kinase reaction proteins were resolved by SDS-PAGE, and the phosphorylation of myelin basic protein was confirmed by phosphorimaging (Molecular Dynamics) and quantitated by Cerenkov counting.

RESULTS AND DISCUSSION

To evaluate the role of the PTK JAK2 in GH-stimulated MAPK activity, we expressed the full-length mGHR and HA-
tagged p42 MAPK (ERK2) in human embryonic kidney (293) cells, in the presence or absence of coexpressed JAK proteins. JAK2 is ubiquitously expressed; however, the level of expression in 293 cells appears to be sufficiently low that some level of overexpression of JAK2 is required in order to detect activation (25). We found the level of immunoprecipitable JAK2 in GHR/JAK2 transfected cells to be approximately 10-fold higher than the level in untransfected cells (data not shown). As shown in Fig. 1A, expression of the GHR alone failed to support GH-stimulated activation of ERK2/MAPK, whereas coexpression of the GHR and wild-type JAK2 resulted in a GH-stimulated decrease in the mobility of a fraction of the expressed ERK2/MAPK, which is known to reflect the dual phosphorylation and activation of the enzyme. We confirmed that GH stimulates ERK2/MAPK activity by assaying HA-ERK2/MAPK immunoprecipitates from cells coexpressing the GHR and different JAKs for myelin basic protein (MBP) phosphorylating activity. The observed GH-stimulated increase in activity in GHR/JAK2-expressing cells was approximately 4-fold (Fig. 1B). Wild-type JAK2 expressed in the absence of the GHR did not activate this pathway (data not shown). JAK2 kinase activity was required for the activation of ERK2/MAPK, since activation failed to occur in GH-treated cells in which GHR was coexpressed with a mutant form of JAK2 (JAK2ki) rendered kinase-inactive by two point mutations in the PTK subdomain VIII (22) (Fig. 1, A and B). This is consistent with the recent report by Frank et al. (26) that a form of JAK2 truncated in the functional C-terminal PTK domain can associate with the GHR but not support a GH-stimulated mobility shift in ERK2/MAPK.
A significant role for JAK1 in GH-stimulated ERK2/MAPK activity seems unlikely from the observations that GH stimulates the tyrosine-phosphorylation of JAK2 but not JAK1 in IM-9 cells (27) and that in HeLa cells transiently expressing receptor chimeras containing cytoplasmic domains of the GHR, EPOR, or gp130, JAK1 failed to coprecipitate with either the GHR or EPOR chimera (28). We found that coexpression of JAK1 with the GHR in 293 cells resulted in only a weak GH-stimulated mobility shift in ERK2/MAPK on immunoblotting (Fig. 1A). In cells coexpressing the GHR and JAK2 the stimulation by GH was less than 2-fold, and the level of GH-stimulated ERK2/MAPK kinase activity was less than 30% of that stimulated by GH in GHRJAK2-transfected cells (Fig. 1B). We tested whether the GH-stimulated ERK/MAPK activation mediated by JAK2 required Ras by coexpressing the GHR and JAK2 in 293 cells with or without coexpression of a dominant negative form of Ras (S17N; Ref. 29). As shown in Fig. 2, A and B, GH stimulation of ERK2/MAPK activity was inhibited by dominant negative Ras (dn Ras). That this was not due to a nonspecific effect of dominant negative Ras on ERK2/MAPK activation is evident from the observation that ERK2/MAPK activity was not inhibited in 293 cells transiently expressing constitutively active MEKK1 (JMEKK; 0.02 μg) in the presence or absence of an expression vector construct for a dominant negative form of Ras (dn Ras; 1.5 μg). All other procedures were as described in the legend for Fig. 1.

Moreover, ERK2/MAPK can be activated in cells expressing a truncated rat GHR in which only four of the 10 cytoplasmic domain tyrosine residues are retained (33), and MAP kinase activation is not inhibited by mutation of two of these tyrosine residues to phenylalanine (34). Thus, the GHR itself may not be the binding site for Ras-activating effectors. Alternative mechanisms for Ras activation are suggested by the findings that GH stimulates the tyrosine phosphorylation of IRS-1 (35) and of Shc proteins (36). GH also promotes an increase in the association of Grb2 with Shc and the association of JAK2 with a GST-Shc SH2 domain fusion protein (36). Although in vivo association of Shc with JAK2 was not detected, the latter finding suggested the possibility that JAK2 itself might provide the phosphotyrosine binding site(s) for effector molecules on the ERK/MAPK signaling pathway (see also below). Indeed, Shc has been shown to associate in vivo with JAK2 in cells expressing a truncated EPOR lacking tyrosine residues (38).

To test the requirement for Raf in GH activation of ERK2/MAPK, the GHR and JAK2 were coexpressed in 293 cells with or without coexpression of a dominant negative form of Raf-1, which harbors a point mutation (K375W) in the ATP binding site (37). Although we found TPA to increase in the association of Grb2 with Shc and the association of JAK2 with a GST-Shc SH2 domain fusion protein (36). Although in vivo association of Shc with JAK2 was not detected, the latter finding suggested the possibility that JAK2 itself might provide the phosphotyrosine binding site(s) for effector molecules on the ERK/MAPK signaling pathway (see also below). Indeed, Shc has been shown to associate in vivo with JAK2 in cells expressing a truncated EPOR lacking tyrosine residues (38).
dependence in 293 cells (39). Activation of ERK2/MAPK by ΔMEKK, on the other hand, was not inhibited by dominant negative Raf (Fig. 3A). Further support for a role of Raf in GH-stimulated ERK2/MAPK activity is suggested by the observation that overexpression of wild-type Raf-1, which when expressed alone did not activate ERK2/MAPK, could reproducibly increase ERK2/MAPK activation when coexpressed with the GHR and J AK2 (Fig. 3C), although the magnitude of this effect varied between experiments (Fig. 3B).

Of relevance to the J AK2-mediated activation of Raf is the recent finding that Raf-1 physically associates with JAK2 in insect cells in which Raf-1 and JAK2 are coexpressed, in EPO-recent finding that Raf-1 physically associates with JAK2 in

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