

in G protein activation, which renders the sperm hypersensitive to their ZP3 ligand. Our results help to explain the relation between the binding of sperm to their ZP3 ligand and the consequent induction of a G protein cascade culminating in the acrosome reaction. Our results also show that surface GalTase does not function simply as a lectin-like cell adhesion molecule, but rather its cytoplasmic domain associates with a signal-transducing network.

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Requirement for MAP Kinase (ERK2) Activity in Interferon α - and Interferon β -Stimulated Gene Expression Through STAT Proteins

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Activation of early response genes by interferons (IFNs) requires tyrosine phosphorylation of STAT (signal transducers and activators of transcription) proteins. It was found that the serine-threonine kinase mitogen-activated protein kinase (MAPK) [specifically, the 42-kilodalton MAPK or extracellular signal-regulated kinase 2 (ERK2)] interacted with the α subunit of IFN- α/β receptor in vitro and in vivo. Treatment of cells with IFN- β induced tyrosine phosphorylation and activation of MAPK and caused MAPK and Stat1 α to coimmunoprecipitate. Furthermore, expression of dominant negative MAPK inhibited IFN- β -induced transcription. Therefore, MAPK appears to regulate IFN- α and IFN- β activation of early response genes by modifying the Jak-STAT signaling cascade.

The Janus family of tyrosine kinases (Jak1, Jak2, Jak3, and Tyk2) are integral components of many cytokine-activated signaling cascades that regulate tyrosine phosphorylation of the STAT proteins such that they can translocate to the nucleus and bind DNA (1, 2). These kinases associate with one or more chains of a given cytokine receptor (2). Tyk2, Jak1, and the Src homology 2 (SH2) domain containing tyrosine phosphatases PTP1C and PTP1D selectively bind to a glutathione-S-transferase (GST) fusion protein that consists of the

membrane-proximal 50 amino acids of the α subunit of the IFN- α/β receptor (3–5). We incubated this IFN- α/β receptor-GST fusion protein coupled to agarose beads with extracts from cells either treated with IFN- β or left untreated. The bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to an Immobilon membrane, and probed with antibodies to phosphotyrosine. A 42-kD tyrosine-phosphorylated protein was detected in extracts prepared from IFN- β -treated cells (Fig. 1A). The membrane was probed with a monoclonal antibody that recognizes all known species of MAPK (Fig. 1A). MAPK from extracts of both untreated and IFN- β -treated cells bound to the GST fusion protein of the IFN- α/β receptor. The 42-kD protein also reacted with a monoclonal antibody specific for the 42-kD form of MAPK (ERK2) (6).

To determine whether MAPK binding was specific for the IFN- α/β receptor, GST

fusion proteins were constructed that contained the entire 100 amino acids of the cytoplasmic domain α subunit of the IFN- α/β receptor, the membrane-proximal or membrane-distal 50 amino acids of the receptor, or GST alone (Fig. 1B). Although Tyk2 bound to both a GST fusion protein containing either the entire cytoplasmic domain of the receptor or the membrane-proximal 50 amino acids, the 42-kD MAPK interacted only with the construct containing the membrane-proximal 50 amino acids. The interaction of MAPK with the 50-amino acid membrane-proximal region, but not the entire cytoplasmic domain, may indicate that the MAPK binding site is not exposed in the full-length fusion protein. The association of MAPK with the α subunit of the IFN- α/β receptor in vivo was independently confirmed by immunoprecipitating the receptor from U266 cells that were either left untreated or treated with IFN- β for various amounts of time. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to Immobilon, and probed for the presence of MAPK (Fig. 1C). The 42-kD MAPK coimmunoprecipitated with the IFN- α/β receptor. Although there was constitutive association of MAPK with the receptor, a more slowly migrating form of MAPK (MAPK-P), that may represent phosphorylated MAPK, was detected 5 to 10 min after incubation of cells with IFN- β .

To directly determine whether activity of MAPK bound to the IFN- α/β receptor-GST fusion protein was enhanced as a result of IFN- β treatment of cells, we assayed MAPK activity by adding myelin basic protein (MBP) and [γ - 32 P]ATP (adenosine triphosphate) to GST fusion protein beads that had been incubated with extracts prepared from either untreated or IFN- β -treated cells (Fig. 2A). The amount of 32 P incorporated into MBP was about five times higher when cells

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were treated with IFN- β . There was no enhanced incorporation into MBP when agarose beads with GST alone were incubated with MBP and [γ - 32 P]ATP. The same membrane was probed with antibodies to MAPK to ensure that equal amounts of MAPK were bound to the fusion proteins incubated in extracts prepared from untreated or IFN- β -treated cells (Fig. 2A). In-gel MBP kinase assays were also done (Fig. 2B) (7). Cells were left untreated or treated with IFN- β , and then either (i) the 42-kD MAPK was directly immunoprecipitated, (ii) the MAPK bound to the GST fusion protein of the IFN- α/β receptor was eluted and then immunoprecipitated with antiserum specific for 42-kD MAPK, or (iii)

the proteins bound to the GST fusion protein were assayed without immunoprecipitation. IFN- β treatment of U266 cells increased the activity of the 42-kD MAPK as measured by all three procedures.

Examination of the protein sequences of the cloned STATs revealed that Stat1, Stat3, and Stat4 contain a single, highly conserved MAPK phosphorylation site (PX_nS/TP; P, proline; S/T, serine or threonine; X, any amino acid; and n = 1 or 2) in the COOH- terminus of the proteins. For Stat1 α the serine is located at amino acid 727. To determine whether the 42-kD MAPK and Stat1 α associate as a result of IFN treatment of cells, antiserum specific

for the 42-kD form of MAPK (8) was added to extracts prepared from either untreated or IFN- β -treated cells. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to Immobilon, and probed with either an antibody to Stat1 or a monoclonal antibody to MAPK (Fig. 3A). Treatment of cells with IFN- β for various amounts of time revealed a time-dependent association of Stat1 α with MAPK. In an analogous manner, lysates from IFN- β -treated cells were subjected to immunoprecipitation with Stat1 α antiserum; immunoblots showed a ligand-dependent association of MAPK with Stat1 α (Fig. 3B).

The fact that an association between MAPK and Stat1 α was detected suggested that the MAPK pathway and the Jak-STAT pathway may be linked, so we next examined the role of MAPK in IFN activation of early response genes that require activation of the Jak-STAT pathway. We used transient transfection assays to determine whether expression of dominant negative 42-kD MAPK affected IFN- β activation of a luciferase reporter containing the interferon-stimulated response element (ISRE) (9). These experiments were done with primary human fibroblasts because U266 cells could not be efficiently transfected and MAPK also associated with the GST fusion protein in primary fibroblasts (6). Fibroblasts were either transfected with the luciferase construct alone or with plasmids encoding either wild-type MAPK or MAPK in which the threonine and tyrosine residues that are re-

Fig. 1. Interaction of proteins with the cytoplasmic domain of the α subunit of the IFN- α/β receptor. U266 cells (1×10^6 to 2×10^6 cells per sample) were incubated for 10 min at 37°C with or without (CTL) recombinant human IFN- β (10^3 units/ml), and cell extracts were prepared as described (13). **(A)** GST fusion proteins containing the membrane-proximal 50 amino acids of the cytoplasmic tail (465) of the IFN- α/β receptor (4) bound to agarose beads were incubated with the cell extracts at 4°C for 2 to 12 hours, sedimented at 15,000g for 2 min, and washed three times with ice-cold lysis buffer (1 ml). Proteins were separated by SDS-PAGE (4 to 16% gels) and transferred to Immobilon (Millipore). The membranes were then incubated with monoclonal antibodies (mAbs) to phosphotyrosine (PY20; ICN, Irvine, CA) (anti-PY) (lanes 1 and 2), MAPK [lanes 3 and 4, and (B), lower panel], or Tyk2 [(B), upper panel]. Antibodies were detected by enhanced chemiluminescence. All mAbs except PY20 were obtained from Transduction Laboratories. **(B)** Either GST alone (lanes 1 and 2) or GST fusion proteins containing the entire coding region of the cytoplasmic domain of the α subunit of the IFN- α/β receptor (α R) (lanes 3 and 4), the COOH-terminal 50 amino acids (511) (lanes 5 and 6), or the membrane-proximal 50 amino acids of the cytoplasmic tail (465) (lanes 7 and 8) were bound to the beads. Expression of the fusion proteins has been described (4). **(C)** U266 cells were incubated without IFN- β (CTL) or with IFN- β (10^3 units/ml) for 2, 5, or 10 min. Cellular extracts were prepared (15), and proteins were immunoprecipitated with mAbs to the extracellular domain of the IFN- α/β receptor (IFN- α/β R) (14). The immunoprecipitates were resolved by SDS-PAGE, and the proteins transferred to Immobilon and probed with mAb to MAPK. MAPK-P, a form of MAPK which may be phosphorylated.

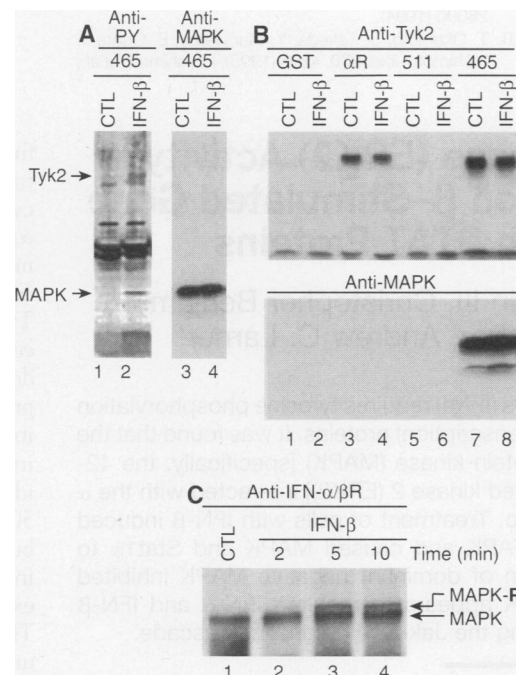


Fig. 2. Increased activity of MAPK in U266 cells treated with IFN- β . **(A)** Activation of MAPK bound to the IFN- α/β receptor-GST fusion protein. Cellular extracts were prepared (13) from U266 cells either untreated (CTL) or treated with IFN- β as described in Fig. 1. Extracts were then incubated with GST fusion proteins bound to agarose beads. The beads were washed and then incubated at 30°C for 20 min with MBP (20 μ g) in a buffer (16). SDS sample buffer was added to terminate the reaction. The proteins were separated by SDS-PAGE, transferred to Immobilon, and autoradiography performed (top panel). The Immobilon was then probed with mAb to MAPK (bottom panel). **(B)** In-gel MBP kinase assay. U266 cells were treated with IFN- β or left untreated (CTL) and cellular extracts prepared as described (Fig. 1A). MAPK (42-kD) was either directly immunoprecipitated (TR10) (lanes 1 and 2), bound to GST fusion protein (465) and then immunoprecipitated with antiserum to MAPK (TR10) (lanes 3 and 4), or the material bound to the beads was assayed directly (lanes 5 and 6). All samples were resolved by SDS-PAGE on gels polymerized with MBP (0.4 mg/ml). Assays done as described (7).

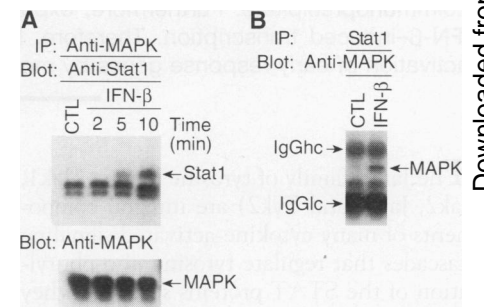
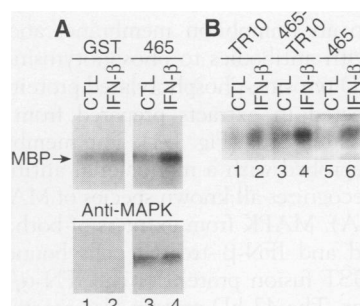
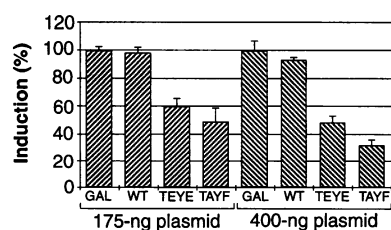


Fig. 3. Association of MAPK with Stat1 α . **(A)** Coimmunoprecipitation of Stat1 α with antiserum to 42-kD MAPK. Extracts from either untreated cells (CTL) (lane 1) or cells incubated with IFN- β for 2, 5, or 10 min were prepared and incubated with antiserum specific to MAPK (TR10) (8). Immunoprecipitates (IP) were resolved by SDS-PAGE and transferred to Immobilon. The membranes were probed for the presence of Stat1 α (upper panel) or MAPK (lower panel) as described. **(B)** Stat1 α antiserum coimmunoprecipitates MAPK. The same experiment as in (A) was done except that cells were incubated with IFN- β for 5 min, and the cellular extracts were immunoprecipitated with antiserum to Stat1 α . The resulting blot was then probed for the presence of MAPK. An equal amount of Stat1 α was present in both samples (6). IgGhc, immunoglobulin G heavy chain; IgGlc, IgG light chain.

Fig. 4. Inhibition of IFN- β -stimulated transcription of an ISRE reporter plasmid in cells expressing dominant negative 42-kD MAPK. Primary human fibroblasts were transfected as described (17) with an ISRE derived from the IFN-stimulated responsive gene ISG15 linked 5' to a thymidine kinase minimal promoter-luciferase reporter (9). In addition to the reporter construct, plasmids containing complementary DNAs (cDNAs) corresponding to either wild-type MAPK (WT) or MAPK in which Thr¹⁸³ and Tyr¹⁸⁵ were replaced with either glutamic acid (TEYE) or alanine and phenylalanine (TAYF). These plasmids all contained a cytomegalovirus (CMV) promoter. To normalize for DNA in the transfection, a CMV-driven β -galactosidase cDNA (GAL) was included when the MAPK kinase plasmids were not present. The plasmids were used at either 175 ng/ml or 400 ng/ml in the transfection. Twenty hours after transfection, IFN- β (10^3 units/ml) was added to the cells for 6 hours before preparation of cell lysates and assay of luciferase activity. The amount of luciferase activity in the cells transfected with β -galactosidase and treated with IFN- β (which was consistently five- to sixfold that of untreated cells) was normalized to 100%. The reporter construct without the ISRE showed no increased luciferase activity in the presence of IFN- β . The standard deviations for these experiments are denoted by error bars.



quired to be phosphorylated for activity were replaced with either glutamic acid (TEYE) or alanine and phenylalanine (TAYF), rendering the proteins inactive (8). After transfection fibroblasts were incubated with or without IFN- β , the cells lysed, and luciferase activity assayed. Consistently, IFN- β stimulated a five- to sixfold increase in the luciferase activity in cells transfected with the ISRE containing the luciferase reporter. Co-transfection of wild-type MAPK with the reporter had little if any effect on IFN- β -stimulated luciferase activity, whereas the mutant MAPK constructs showed a dose-dependent inhibition of IFN- β -stimulated luciferase activity (Fig. 4). Thus, it appears that MAPK participates in the mechanism by which IFN- β stimulates early response genes that require activation of the STAT transcription factors.

Interleukin 6 induces serine phosphorylation of Stat3, which is required for binding of Stat3 homodimers to DNA (10). Although we have not observed a requirement of serine phosphorylation for ISRE binding of Stat1 α , treatment of U266 cells with IFN- β does stimulate serine phosphorylation of the protein (11). The serine-threonine kinase inhibitor H7 can selectively inhibit IFN- α activation of ISRE-dependent genes without affecting Stat1 and Stat2 binding to the ISRE (6, 12). These data, together with those of Zhang *et al.* (10), would suggest that IFN- α - or IFN- β -stimulated phosphorylation of both serine and tyrosine on Stat1 α is required for transactivation.

It remains to be established whether activation of MAPK is required to stimulate tyrosine phosphorylation of Stat1 and Stat2 by IFN- α and IFN- β . Our results indicate that one target of MAPK is a conserved phosphorylation site in the COOH terminus of Stat1 α , and possibly Stat3 and Stat4. Mapping of the interaction sites between the 42-kD MAPK and the STAT proteins will elucidate the role of MAPK in transcriptional activation of STAT-responsive genes.

Note added in proof: Recently, Wen *et al.* (18) reported that Ser⁷²⁷ in Stat1 is phosphorylated. Maximal transcriptional activation by IFN- γ requires that this serine be present in Stat1.

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13. Cells were diluted with ice-cold phosphate-buffered saline (PBS) and centrifuged at 1500g for 10 min at 4°C, washed with PBS, and resuspended in lysis buffer containing 20 mM Hepes (pH 7.4), 1 mM vanadate, 10 mM β -glycero-phosphate, 100 mM NaCl, 50 mM NaF, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The lysate was mixed, incubated on ice for 10 min, and centrifuged at 18,000g for 10 min at 4°C.
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Imaging Elementary Events of Calcium Release in Skeletal Muscle Cells

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In skeletal muscle cells, calcium release to trigger contraction occurs at triads, specialized junctions where sarcoplasmic reticulum channels are opened by voltage sensors in the transverse tubule. Scanning confocal microscopy was used in cells under voltage clamp to measure the concentration of intracellular calcium, [Ca²⁺]_i, at individual triads and [Ca²⁺]_i gradients that were proportional to calcium release. In cells stimulated with small depolarizations, the [Ca²⁺]_i gradients broke down into elementary events, corresponding to single-channel currents of about 0.1 picoampere. Because these events were one-tenth to one-fifth the size of calcium sparks (elementary release events of cardiac muscle), skeletal muscle control mechanisms appear to be fundamentally different.

For muscles to contract, Ca²⁺ must be released from the sarcoplasmic reticulum (SR) rapidly and massively, through channels of the ryanodine receptor family (1) present also in neurons (2), endothelial (3), and other cells (4). In cardiac muscle, Ca²⁺ release is determined by the membrane potential-dependent entry of Ca²⁺ (5), whereas in skeletal muscle release is con-

trolled by membrane potential without Ca²⁺ entry (6, 7). In confocal images of Ca²⁺ from voltage-stimulated skeletal muscle fibers, we found elementary events of release apparently derived from the opening of single SR channels.

We adapted the single muscle fiber voltage clamp (8, 9) to work synchronously with a laser scanning confocal microscope. In a cell at rest (-90 mV) containing the Ca²⁺ indicator fluo 3, the banded pattern of fluorescence indicated greater access or binding of fluo 3 to specific regions of the sarcomere (Fig. 1A). Fluorescence along a line was scanned every 1.39 ms and successive scans plotted in a line scan image (Fig. 1B). The periodic dye distribution resulted

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