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Activation of a G Protein Complex by Aggregation of β-1,4-Galactosyltransferase on the Surface of Sperm

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Fertilization is initiated by the species-specific binding of sperm to the extracellular coat of the egg. One sperm receptor for the mouse egg is β-1,4-galactosyltransferase (GalTase), which binds O-linked oligosaccharides on the egg coat glycoprotein ZP3. ZP3 binding induces acrosomal exocytosis through the activation of a pertussis toxin–sensitive heterotrimeric guanine nucleotide–binding protein (G protein). The cytoplasmic domain of sperm surface GalTase bound to and activated a heterotrimeric G protein complex that contained the $G_{i\alpha}$ subunit. Aggregation of GalTase by multivalent ligands elicited G protein activation. Sperm from transgenic mice that overexpressed GalTase had higher rates of G protein activation than did wild-type sperm, which rendered transgenic sperm hypersensitive to their ZP3 ligand. Thus, the cytoplasmic domain of cell surface GalTase appears to enable it to function as a signal-transducing receptor for extracellular oligosaccharide ligands.

The species-specific binding of sperm to the egg coat initiates a series of events that culminates in the formation of the zygote. In the mouse, sperm recognition of the egg coat requires the binding of GalTase on the sperm plasma membrane to O-linked oligosaccharide ligands on the zona pellucida glycoprotein ZP3 (*1*). After egg activation, the block to polyspermy is facilitated by the release of hexosaminidase from the egg cortical granules, which removes the binding site on ZP3 oligosaccharides for sperm GalTase (*1*).

GalTase is a biosynthetic component of the Golgi complex in somatic cells. However, in both somatic cells and sperm, GalTase is also expressed on the cell surface, where it functions as a receptor for extracellular oligosaccharide ligands. GalTase's dual subcellular distribution results, at least in part, from the synthesis of two GalTase isoforms with different cytoplasmic domains (*2*). The shorter isoform, which contains an 11–amino acid cytoplasmic domain, functions biosynthetically in the Golgi complex; the longer isoform contains an additional 13–amino acid sequence that overrides the Golgi retention signal and targets some GalTase molecules to the cell surface. The longer GalTase isoform mediates the binding of sperm to ZP3 oligosaccharides in the zona pellucida and is the only isoform found in mature sperm (*1*, *3*). Two other ZP3-binding proteins have been identified on mouse sperm in addition to GalTase. One appears to be a cell surface protein with intrinsic hexokinase activity (*4*); the other behaves as a peripheral membrane protein (*5*).

The binding of sperm to ZP3 induces the acrosome reaction, in which hydrolytic enzymes are released that aid penetration of sperm through the zona pellucida. The acrosome reaction appears to be induced by multivalent oligosaccharides on ZP3 that aggregate sperm-bound receptors, thus eliciting intracellular signals. ZP3 glycopeptides that bind to sperm are unable to induce the acrosome reaction unless they are cross-linked by antiserum to ZP3 (*6*). Induction of the acrosome reaction by ZP3 is inhibited by pertussis toxin (PTx), which suggests that a G protein complex that contains G_i or G_o is required (*7*). A $G_{i\alpha}$ subunit has been identified in sperm, as assessed by PTx-dependent adenosine diphosphate (ADP) ribosylation and immunoblotting of a 41-kD sperm protein; mature sperm do not appear to contain the G_o subunit (*7*). Other signal transduction pathways elicited by ZP3 or by progesterone (*8*) that involve calcium mobilization (*9*) or phosphorylation of tyrosine residues (*4*) also likely contribute to induction of the acrosome reaction.

Although it is clear that sperm GalTase participates in fertilization by binding glycoside ligands on ZP3, and that ZP3 binding elicits a $G_{i\alpha}$ cascade to induce the acrosome reaction, the relation between sperm GalTase and activation of the $G_{i\alpha}$ cascade remains unknown. We determined whether sperm GalTase binds to and activates a $G_{i\alpha}$ cascade by testing four predictions. Previous studies showed that intact antibodies to sperm GalTase (anti-GalTase) induce the acrosome reaction whereas Fab fragments do not unless they are cross-linked with a secondary antibody (*10*). Thus cross-linking GalTase mimics the effect of ZP3 binding. Because ZP3-induced acrosome reactions are inhibited by PTx (*7*), the first prediction to be tested is that acrosome reactions induced by anti-GalTase might also be sensitive to PTx, which would suggest the involvement of a $G_{i\alpha}$ cascade. Second, if GalTase functions through a PTx-sensitive cascade, then the cytoplasmic domain of GalTase might associate, directly or indirectly, with a $G_{i\alpha}$ subunit as well as with the βγ subunit. Third, because ZP3 binding to sperm membranes induces G protein activation (*11*), the aggregation of sperm GalTase by anti-GalTase should also elicit G protein activation. Finally, transgenic sperm that have elevated amounts of surface GalTase (*12*), and which bind more ZP3 ligand than do wild-type sperm, should show enhanced G protein activation.

We tested the ability of PTx to inhibit acrosomal exocytosis induced by anti-GalTase (Fig. 1). Positive controls included sperm treated with ionophore or with solubilized zona glycoproteins. Anti-GalTase and zona glycoproteins induced the acrosome reaction to a similar degree. Neither preimmune immunoglobulin G (IgG) nor anti-GalTase Fab fragments had any significant effect on acrosomal exocytosis (Fig. 1) (*10*). The addition of PTx inhibited zona glycoprotein–induced and anti-GalTase–induced acrosome reactions to background rates. Because PTx inhibits only "physiological" acrosome reactions, that is, those induced by a ZP3-dependent cascade (*7*), these

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results suggest that GalTase-dependent induction of the acrosome reaction also involves a $G_{i\alpha}$-containing heterotrimer.

To show directly that the cytoplasmic domain of GalTase associates with a $G_{i\alpha}$-containing heterotrimeric complex, we synthesized peptides corresponding to the complete 24–amino acid cytoplasmic domain of the long GalTase isoform (MRFREQFLGGSAAMPGATLQRACR) (*13*). The COOH-terminus was biotinylated to allow precipitation with streptavidin-agarose. Three control peptides were synthesized and biotinylated: the 11–amino acid cytoplasmic domain of the short GalTase isoform (MPGATLQRACR) (*2*) and two peptides composed of the same 24 amino acids of the long GalTase cytoplasmic domain in different scrambled sequences (scrambled sequence I, ARGMGFRAPQLSRLGAMECFARQT; scrambled sequence II, RPAQCMRGTFALRGQAERGFSMLA). The biotinylated peptides were incubated with sperm lysates for at least 3 hours, streptavidin-agarose was added to precipitate the peptides and any associated sperm proteins, and the precipitate was washed four times in buffer.

The presence of the $G_{i\alpha}$ subunit was assessed by PTx-dependent ADP ribosylation (*7*). PTx inhibits $G_{i\alpha}$-mediated signal transduction by ADP ribosylation of the $G_{i\alpha}$ subunit, which is subsequently released from the cytoplasmic domain of the G protein–coupled receptor and can be recovered in the supernatant. The streptavidin-agarose–bound peptides along with any associated sperm proteins were incubated with [^{32}P]nicotinamide adenine dinucleotide (NAD) and PTx. After incubation for 45 min, the streptavidin-agarose–peptide complex was sedimented, washed, and resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). In parallel, [^{32}P]ADP-ribosylated proteins released into the supernatant were precipitated with trichloroacetic acid (TCA) and resolved by SDS-PAGE. As a positive control to identify the relevant $G_{i\alpha}$ subunit, a sample of the original sperm lysate was also incubated with PTx and [^{32}P]NAD.

A [^{32}P]ADP-ribosylated $G_{i\alpha}$ subunit was readily detectable in the sperm lysate (Fig. 2A) (*7*). No [^{32}P]ADP-ribosylated protein was detectable in the absence of PTx. The ^{32}P-labeled protein was also immunoprecipitated with rabbit anti-$G_{i\alpha}$ (C10) and protein A–agarose. Rabbit IgGs to the Gβ subunit (anti–β subunit) (T20) or to the G_o subunit (*14*) failed to immunoprecipitate the [^{32}P]ADP-ribosylated protein. The sperm GalTase cytoplasmic domain also bound a 41-kD $G_{i\alpha}$ protein that was subsequently [^{32}P]ADP-ribosylated in the presence of PTx and [^{32}P]NAD (Fig. 2B). As expected, [^{32}P]ADP ribosylation caused most of the 41-kD protein to be released from the cytoplasmic domain into the supernatant. No [^{32}P]ADP-ribosylated protein was detectable in the absence of peptide (that is, with streptavidin-agarose alone), nor did any of this protein associate with the peptide corresponding to the short GalTase cytoplasmic domain or with either of the peptides that contained scrambled sequences of the long GalTase cytoplasmic domain.

We determined whether the GalTase cytoplasmic domain also bound to the βγ subunit of the heterotrimeric G protein complex. The streptavidin-agarose–bound peptides and associated sperm proteins were resolved by SDS-PAGE and immunoblotted with anti–β subunit. Anti–β subunit recognized a protein of the appropriate molecular size (35 kD) in the sperm lysate. When the sperm proteins that bound to the long GalTase cytoplasmic domain were probed with anti–β subunit, the appropriate β subunit protein was readily identified. No β subunit was found to be associated with any of the control peptides, nor was any β subunit detectable in the absence of peptide.

If the GalTase cytoplasmic domain associates with a $G_{i\alpha}$-containing complex, then

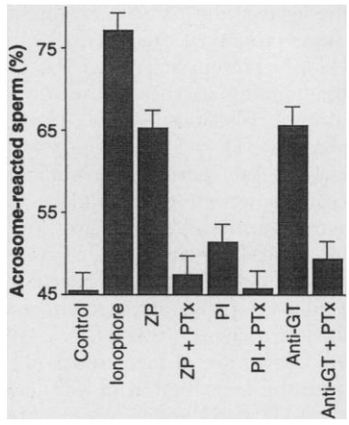

Fig. 1. Inhibition of anti-GalTase–induced acrosome reactions by PTx. Sperm were collected from the cauda epididymis of at least two mice and capacitated (*1*). After washing, the sperm were resuspended in a modified Krebs-Ringer bicarbonate solution (*1*) (2×10^7 sperm/ml). The indicated reagents were added to 0.4×10^6 sperm in a total volume of 40 μl. Calcium ionophore (A23187, 10 μM) was added from a stock (10 mM) in dimethyl sulfoxide. PTx (Gibco-BRL; final concentration 100 ng/ml) was added from a stock (50 mg/ml). Anti-GalTase (anti-GT) and preimmune IgG (PI) (*10*) were added at concentrations of 20, 80 (shown), or 200 μg/ml; all three concentrations produced similar results. Solubilized zona glycoproteins (ZP) were added to a final concentration of 80 ng/μl. After addition of all reagents, samples were incubated for 1 hour at 37°C. Sperm were fixed for 10 min at room temperature in 4% paraformaldehyde, centrifuged, washed once in 0.1 M ammonium acetate, and air-dried on microscope slides. The slides were stained with 0.22% Coomassie Blue G-250 in 50% methanol and 10% acetic acid, rinsed with water, covered with glycerol and phosphate-buffered saline, and sealed with nail polish. At least 100 sperm were scored from each incubation tube. Assays were done in triplicate; the results are averages of three separate experiments. Data were analyzed by analysis of variance and specific comparisons were tested with Tukey's test. Error bars represent pooled SEM from the three experiments.

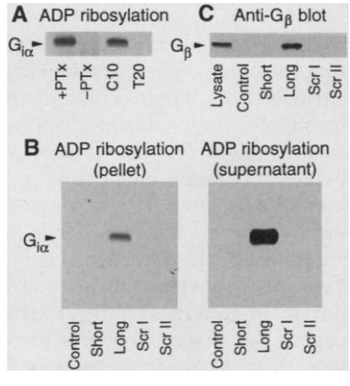

Fig. 2. Association of the cytoplasmic domain of sperm GalTase with a $G_{i\alpha}$-containing heterotrimeric complex. Sperm from 20 CD1 mice were collected and capacitated (*1*). Washed sperm were extracted for 1 hour on ice in 3.2 ml of tris-buffered saline (TBS, pH 7.5) containing 0.5% Triton X-100, 0.5% NP-40, and a protease inhibitor cocktail (PIC) (*21*). The supernatant was collected by centrifugation; 250 μl of the supernatant was incubated with biotinylated peptide (12 μg) for at least 3 hours at 4°C. The peptides were precipitated by addition of 35 μl of 50% streptavidin-agarose. The precipitate was washed by centrifugation in TBS containing 0.1% Triton X-100 and analyzed for the presence of either the $G_{i\alpha}$ or βγ subunits (*11*). (**A** and **B**) Detection of the $G_{i\alpha}$ subunit. The precipitate was resuspended in buffer (*11*) containing activated PTx (20 μg/ml) (*22*) and 5 μM [^{32}P]NAD (ICN Biomedicals, Irvine, California). After incubation for 45 min at 30°C, the supernatants were collected by centrifugation and the soluble proteins were precipitated with TCA. The streptavidin-agarose–peptide complex and the TCA-precipitated proteins were boiled in sample buffer and resolved by SDS-PAGE and autoradiography. The autoradiogram is representative of three independent assays. (**C**) Detection of the βγ subunit. The washed precipitate was resuspended in sample buffer, boiled, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose, and the blot was probed with rabbit anti–β subunit (T20; Santa Cruz Biotechnology, Santa Cruz, California), which was detected by ECL chemiluminescence (Amersham). The autoradiogram is representative of five independent assays. Peptides were synthesized and biotinylated at the COOH-terminus in the Peptide Synthesis Facility at M. D. Anderson Cancer Center. C10, rabbit IgG to $G_{i\alpha 1\text{-}3}$ (Santa Cruz Biotechnology); T20, rabbit IgG to G_β (Santa Cruz Biotechnology); Lysate, sperm lysate; Control, no peptide (streptavidin-agarose alone); Short and Long, the GalTase cytoplasmic domains (MPGATLQRACR and MRFREQFLGGSAAMPGATLQRACR, respectively); Scr I, scrambled sequence I (ARGMGFRAPQLSRLGAMECFARQT); and Scr II, scrambled sequence II (RPAQCMRGTFALRGQAERGFSMLA). Arrowheads indicate the position of the endogenous G protein subunit in the sperm lysate.

the aggregation of GalTase might activate the G protein cascade, as does ZP3 binding (*11*). G protein activation was assessed by the binding of the nonhydrolyzable substrate [^{35}S]guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) to isolated sperm membranes. As a positive control, zona glycoproteins were used to induce G protein activation. In all experiments, the addition of 1 mM unlabeled GTP-γ-S reduced [^{35}S]GTP-γ-S binding to background rates. Addition of zona glycoproteins produced a modest increase (~25%) in [^{35}S]GTP-γ-S binding to sperm membranes (*11*). To reduce the contribution of ZP3-independent [^{35}S]GTP-γ-S binding, we incubated sperm membranes with 0.1 μM unlabeled GTP-γ-S for 15 min, after which the mixture was diluted three times before the addition of zona glycoproteins and [^{35}S]GTP-γ-S. Under these conditions, the amount of [^{35}S]GTP-γ-S bound to sperm membranes approximately doubled in response to the addition of zona glycoproteins (Fig. 3). Treatment of sperm membranes with anti-GalTase according to the same protocol induced about the same amount of [^{35}S]GTP-γ-S binding as did zona glycoproteins. Anti-GalTase was isolated from rabbits immunized with bacterially expressed recombinant murine GalTase and is specific for the murine GalTase polypeptide backbone (*15*). Preimmune IgG from the same rabbit had no effect on [^{35}S]GTP-γ-S binding, nor did zona glycoproteins from 2-cell embryos that had lost their sperm-binding activity (*1*). Thus, the aggregation of GalTase by anti-GalTase leads to G protein activation as assessed by [^{35}S]GTP-γ-S binding.

We tested whether sperm from transgenic mice that overexpress GalTase had an enhanced capacity for G protein activation. Sperm from these mice bind approximately twice as much ZP3 ligand as do wild-type sperm (*12*). Sperm membranes from transgenic mice bound approximately three times as much [^{35}S]GTP-γ-S as did membranes from wild-type mice (Fig. 4A). This finding is consistent with the observation that sperm from these transgenic mice show higher rates of spontaneous acrosome reactions than do wild-type sperm (*12*). The addition of ZP3 produced a further increase in [^{35}S]GTP-γ-S binding (Fig. 4B), consistent with the fact that these sperm are hypersensitive to ZP3 ligand as assessed by higher rates of acrosome reactions in response to ZP3 binding (*12*). This hypersensitivity to ZP3 is most easily attributed to the increased G protein activation that is characteristic of these sperm.

G proteins traditionally associate with transmembrane receptors that contain seven membrane-spanning domains. However, a number of single-spanning transmembrane receptors have been reported to activate G protein cascades (*16*), and in at least one case a direct association has been demonstrated between a single-spanning membrane receptor and the $G_{i\alpha}$-protein complex (*17*). Although there is some debate regarding this issue (*18*), the G protein–activating domain within these receptors appears to be a peptide of 14 to 18 amino acids that is adjacent to or near the transmembrane domain, is able to function in either orientation, and contains one or two characteristic basic amino acid motifs (*16, 19*). Moreover, phosphorylation of threonine residues within this sequence alters its ability to activate the G protein cascade (*19*). These features have some interesting parallels with GalTase. Because only the long GalTase cytoplasmic domain is able to associate with the $G_{i\alpha}$-protein complex, the 13–amino acid extension unique to the long GalTase isoform must contain critical amino acid residues. The Arg-Phe-Arg motif near the NH_2-terminal cytoplasmic tail (*2*) may be partly responsible. The 13–amino acid sequence likely functions in the context of the complete 24–amino acid cytoplasmic domain, because we were unable to detect $G_{i\alpha}$ binding to the NH_2-terminal 13–amino acid peptide in the absence of the COOH-terminal 11–amino acid sequence (*14*). It is also unknown whether the cytoplasmic domain binds to the $G_{i\alpha}$ subunit directly or requires coupling proteins present in the sperm lysate. The cytoplasmic domain of GalTase contains serine and threonine residues, one or both of which are phosphorylated in response to ligand binding (*14, 20*), which raises the possibility that phosphorylation may regulate G protein association, activation, or both.

Collectively, these results show that sperm GalTase associates with a $G_{i\alpha}$-containing G protein complex and that the aggregation of GalTase leads to G protein activation. Increasing the amount of GalTase produces a corresponding increase

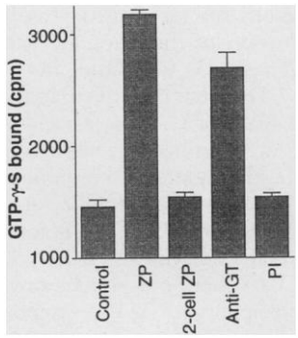

Fig. 3. Activation of G proteins in sperm membranes treated with anti-GalTase. Sperm were collected from CD1 mice, and membranes were prepared (*11*). Membranes (900 μl, 20 to 100 μg/ml) were incubated with 0.1 μM GTP-γ-S (Sigma) (in 1 mM EDTA, 10 mM Mg^{2+}, 1 mM guanosine diphosphate, and 4 mM dithiothreitol) for 15 min at 30°C, after which the preparation was diluted with two additional volumes of buffer. Anti-GalTase (25 μl, 2 to 25 μg) or zona glycoproteins (five zona equivalents; ~20 ng protein) was added to membranes (50 μl) and incubated for another 15 min at 30°C. Buffer (25 μl) containing [^{35}S]GTP-γ-S (DuPont-NEN, Wilmington, Delaware) was added (final concentration 20 nM) and the mixture was incubated for 15 min at 30°C. The membranes were filtered through nitrocellulose and washed with buffer, and radioactivity was counted. Anti-GalTase (anti-GT) was isolated from rabbits immunized with bacterially expressed recombinant murine GalTase (*15*). Preimmune IgG (PI) was isolated from the same rabbit before immunization. Anti-GalTase was assayed at 2, 3, 8, 10, and 25 μg/ml; each assay produced similar results. The data represent seven independent assays; each data point represents a triplicate measurement. Bars represent SEM.

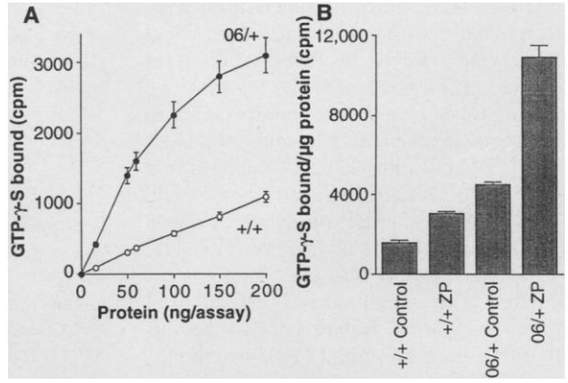

Fig. 4. Increased G protein activation in sperm from transgenic mice that overexpress GalTase. Sperm membranes were isolated from wild-type (+/+) and transgenic (06/+) mice that constitutively overexpress the long isoform of GalTase (*12*) and were assayed for [^{35}S]GTP-γ-S binding. (**A**) Various concentrations of sperm membranes were incubated in 20 nM [^{35}S]GTP-γ-S for 15 min at 30°C; they were then filtered and washed and the amount of associated radioactivity was determined (*11*). The graph represents the least-mean-squares fit of two independent assays; each data point represents a triplicate measurement. Bars represent the average SEM of the two assays. (**B**) Sperm membranes were assayed as described (Fig. 3), including incubation with unlabeled GTP-γ-S to reduce ZP3-independent binding. [^{35}S]GTP-γ-S binding was then assayed in the absence (control) or presence (ZP) of zona glycoproteins. The specific activities (counts per minute per microgram of protein) differ between (A) and (B) because of the incubation with 0.1 μM unlabeled GTP-γ-S in (B). In all experiments, addition of 1 mM unlabeled GTP-γ-S reduced [^{35}S]GTP-γ-S binding to background rates. The results are representative of five independent assays; each data point represents a triplicate measurement. Bars represent SEM.

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 Stat1α 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 reprobed 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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