Interaction of MAD2 with the Carboxyl Terminus of the Insulin Receptor but Not with the IGFIR

EVIDENCE FOR RELEASE FROM THE INSULIN RECEPTOR AFTER ACTIVATION*

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We have utilized the yeast two-hybrid system to identify proteins that interact with the cytoplasmic domain of the insulin receptor (IR). We identified a human cDNA encoding a protein that appears to be the human homolog of the yeast MAD2 protein, which we term hMAD2. The yeast MAD2 protein was first identified in a genetic screen to identify cell cycle checkpoint regulatory proteins, yet the mechanism by which MAD2 functions in cell cycle control is currently unclear. Here we show that hMAD2 requires the COOH-terminal 30 amino acids of the IR for interaction and that hMAD2 does not interact with the related insulin-like growth factor 1 receptor. Interestingly, hMAD2 does not require IR tyrosine autophosphorylation for interaction because it interacts with a kinase-dead IR in the yeast two-hybrid system. In support of this finding, hMAD2-GST fusions were found to interact strongly in vitro with receptors derived from noninsulin-stimulated cells. Furthermore, using two independent in vitro assays, IR activation was found to significantly reduce the interaction of hMAD2 with the IR. Lastly, we show that hMAD2 can be coinmunoprecipitated with the IR from Chinese hamster ovary IR cell lysates, suggesting that this interaction occurs in vivo in cells of mammalian origin. Our results suggest that hMAD2 represents a novel class of proteins that is specific for interaction with the IR as compared with the insulin-like growth factor 1 receptor and that interacts best with the inactive IR and is released upon receptor autophosphorylation. The function of hMAD2 and its potential role in insulin signaling remain to be elucidated.

Insulin regulates a large number of diverse effects within its target tissues. These effects include regulation of metabolic responses such as glucose transporter translocation and regulation of metabolic enzymes such as glycogen synthetase (1). In addition, many other cellular effects are thought to be regulated by insulin, including regulation of amino acid uptake, regulation of a number of enzymes involved in protein and lipid synthesis, and regulation of ion transport (1, 2). These effects are mediated through the insulin receptor (IR) tyrosine kinase. Like other receptor tyrosine kinases, the IR becomes phosphorylated upon tyrosines after insulin binding via an autophosphorylation cascade (3). Phosphorylation of particular tyrosines within the IR leads to the formation of binding sites for a variety of substrates and effector proteins. The best studied of these is the insulin receptor substrate 1 (IRS-1) (4, 5), which interacts with the juxtamembrane domain of the IR containing an NPXY motif. This interaction requires phosphorylation of the tyrosine located within the NPXY motif (amino acid 960), which has been shown to be critical for many insulin-dependent effects (6). Interaction of IRS-1 with the NPXY motif depends upon the phosphotyrosine binding domain located within the amino terminus of IRS-1 (7–9). Interaction of IRS-1 with the IR is believed to result in the phosphorylation of multiple tyrosine residues within IRS-1, leading to subsequent activation of a number of SH2 domain-containing proteins including phosphatidylinositol 3-kinase, GRB2, and the Syp tyrosine phosphatase (10–12). Another substrate of the IR is the “src and collagen homology” protein SHC (13). This protein also interacts with the IR NPXY motif via the phosphotyrosine binding domain of SHC (8, 14). This leads to the interaction of SHC with GRB2 leading to activation of Ras via intermediate nucleotide releasing proteins such as SOS (15–18). A third substrate of the IR is the recently identified IRS-2 protein, which appears to act in a manner similar to IRS-1 (19).

Whether or not the known substrates of the insulin receptor are sufficient to explain insulin-mediated signal transduction is a matter of much interest. Several lines of evidence suggest that there may be additional signaling pathways that may mediate some of insulin’s complex pleiotropic effects. First, recent work has shown that many other receptors including a number of cytokine receptors can also recruit and activate the IRS and SHC proteins (19–22) yet do not appear to act in insulin-like manners (23), suggesting that IR signaling may require additional, more specific signaling pathways. Secondly, the closely related IGFIR, which is not considered to be a physiologically important regulator of metabolic responses, has been shown to interact equally well with the known IR substrates (7, 8, 14, 24–26), suggesting that the IR may activate distinct pathways. To begin to explore the possibility that additional effector proteins may exist that are relatively specific for the IR, we designed experiments to allow the identification

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1 The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; SH2, src homology 2; IGFIR, insulin-like growth factor I receptor; GST, glutathione S-transferase; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; MAP, mitogen-activated protein.

2 The numbering of amino acids of the IR correspond to the sequence of the receptor of Ullrich et al. (54). These differ from that of Ebina et al. (55) by being 12 amino acids less.
and cloning of cDNAs whose protein products interact directly with the IR. Here we report the cloning and identification of a protein that interacts specifically with the IR and not the IGFR. This protein, which we term hMAD2, is related to the yeast MAD2 protein, which plays an uncharacterized role in mitotic cell cycle checkpoint control. We show that the hMAD2 protein interacts with the COOH terminus of the IR in vitro and in cells. Furthermore, hMAD2 interacts well with the unphosphorylated IR and appears to be released upon receptor activation. These findings suggest a potential role for hMAD2 in IR signaling.

MATERIALS AND METHODS

Yeast Strains and Plasmids—The yeast strains EGY191 and EGY48 (alpha, trp1, ara3–52, his3, leu2) and all yeast expression plasmids were provided by the laboratory of Roger Brent and have been previously described (7, 27–29). All routine growth and maintenance of yeast strains were as described (30). The prototrophy assays shown in Fig. 2B utilized the EGY191 strain. Plasmid transformation of yeast was by the lithium acetate method (31). The IR, IGFR, and IR-K1018A and IR-X30 cDNA constructs have been previously reported (7, 8, 14, 24, 25). The IR COOH-terminal Tyr site-directed mutants were generated using the methods of Kunkel (32) using customized primers. The IR-IGFR chimera was derived from the chimeras originally described by LeRoith and colleagues (33). The truncated MAD2 hybrid proteins (see Fig. 1C) were generated using the following restriction sites within MAD2: 2–136 (BcI), 2–63 (SacI), and 45–205 (XhoI). Detailed cloning strategies for all clones are available upon request.

β-Galactosidase Assays—The colony color β-galactosidase assay was performed as described (7, 34). The solution β-galactosidase assays were performed as described (35), and the units of β-galactosidase activity were calculated by the method of Miller (36).

Northern Blot Analysis—Human RNA blots were purchased from Clontech and hybridized to a hMAD2 probe using standard methodologies. The probe used corresponded to amino acid 12 to the 3′ end of the cDNA.

Two-hybrid Library Screening—We screened a HeLa cDNA library provided by the Brent laboratory using the interaction trap methodology with the IR cytoplasmic domain as bait (27, 29). We termed these proteins GRIPs for growth factor receptor interacting proteins. The plasmids encoding the GRIP proteins were isolated from yeast and transferred to DH5α bacteria prior to sequencing. The initial hMAD2 clone contained all of the cDNA except the initial 12 amino acids. We obtained the sequence of the remaining 12 amino acids from Robert Benezra (Sloan Kettering), who had also cloned this cDNA (37).

In Vitro Interaction Studies—In these studies, we generated a GST fusion protein by introducing amino acids 13–205 of hMAD2 into the GST vector pGEX5X (Pharmacia Biotech Inc.). This fusion protein was expressed in bacteria and purified onto glutathione-agarose beads by standard methods (38). The beads that contained immobilized fusion protein were then incubated with cell lysates derived from CHO-IR cells (39) (which overexpress the IR) prior to or after insulin stimulation (10 min, 100 nm). Lysates were prepared by lysis for 30 min on ice in 50 mM HEPES (pH 7.6), 1% Triton X-100, 1 mM EGTA, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml of aprotinin and leupeptin followed by spinning at 10,000 × g for 10 min to remove insoluble material. The resulting lysate supernatants were incubated with the immobilized GST proteins for 4 h. After extensive washing with 50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, the proteins that coprecipitated with the hMAD2, Grb10 (26) or control GST proteins were analyzed by SDS-PAGE followed by immunoblotting with either anti-IR (IR-CT1) (a gift from Ken Siddle) or anti-phosphotyrosine antibodies (PY20) (Transduction Labs).

In another set of in vitro interaction experiments, we overexpressed the cytoplasmic domains of the IR and IGFR as histidine-tagged fusion proteins in baculoviral expression systems. After purification of these proteins to >90% homogeneity (data not shown), the receptor domains were stimulated to autophosphorylate in vitro by the addition of ATP (10 mM) and MnCl2 (10 mM) for 60 min at 20 °C or alternatively were incubated without ATP. These proteins were then separated using 10% SDS-PAGE and blotted to nitrocellulose. After blocking the filters with 3% dry milk in phosphate-buffered saline (2 h at room temp) the filters were probed with GST fusion proteins that contained a protein kinase A phosphorylation site (40) and could therefore be labeled to high specific activity using protein kinase A purified from bovine heart muscle (Sigma). These GST fusions were labeled as described previously while bound to glutathione-agarose beads, the free 32P was washed away, and the labeled proteins were eluted from the beads with glutathione prior to use as probes (40).

RESULTS

Identification of hMAD2 Using the Yeast Two-hybrid System—We screened a HeLa cell-derived cDNA library with the intact IR cytoplasmic domain as the LexA (bait) hybrid (7). The cDNAs that were identified in this assay were termed GRIPs for growth factor receptor interacting proteins. One of these (GRIP 3) was found to be homologous to the MAD2 protein that had been previously described in the budding yeast Saccharomyces cerevisiae (41). Our initial cDNA encoded amino acids 13–205 of a protein of 205 amino acids. The remaining sequence was generously supplied to us by Robert Benezra prior to publication (37). As shown in Fig. 1A, the hMAD2 protein is ~40% identical and ~80% similar to the yeast protein. Neither protein had any identifiable structural or functional motifs, and data base searches did not reveal any other closely related proteins. As shown in Fig. 1B, Northern blot analysis of a human tissue blot revealed that hMAD2 hybridized to a single band of ~1.5 kilobases whose expression was fairly ubiquitous.

FIG. 1. Identification of the human MAD2 homolog and its interaction with the insulin receptor. A, we screened a HeLa cell two-hybrid cDNA library using the cytoplasmic domain of the IR as “bait.” We identified the hMAD2 protein as an interactor of the IR. The hMAD2 amino acid sequence is shown aligned to the yeast MAD2 protein. B, expression pattern of hMAD2 mRNA in human tissues. H, heart; Br, brain; Pl, placenta; Lu, lung; Li, liver; Sk, skeletal muscle; K, kidney; Pa, pancreas. C, interaction of truncated hMAD2 proteins with the IR in the two-hybrid assay. The (+ + +) refers to dark blue colonies, and (−) refers to white colonies.
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Fig. 3. In vitro interaction of hMAD2 and the IR. We generated a hMAD2-GST fusion protein that was immobilized upon glutathione-agarose beads. These beads were incubated with lysates derived from CHO-IR cells that were either unstimulated or stimulated with insulin (10 min, 100 nM) prior to lysate production. After incubation with the lysates, the beads were washed extensively, and the coprecipitating proteins were analyzed by immunoblot analysis using either an IR antibody (αIR.CT1) or phosphotyrosine antibody (αPY20).

In Vitro Interaction of hMAD2 and the IR—To further test the interaction between the IR and hMAD2, we produced a hMAD2-GST fusion protein using amino acids 13–205 of hMAD2. We utilized this fusion protein in GST “pull-down” assays in which we added the immobilized GST fusions to lysates from CHO-IR cells (39) (which overexpress the IR) that were either unstimulated or stimulated with insulin to activate IR. To assess interaction with the IR in vitro, we analyzed proteins that coprecipitated with these GST fusions by subsequent immunoblotting with anti-IR or antiphosphotyrosine antibodies. As shown in Fig. 3, the IR was easily coprecipitated with hMAD2-GST fusion protein using amino acids 13–205 of hMAD2. We therefore mutated these residues to phenylalanine either singly or doubly. As shown in Fig. 2 (B and C), these mutations had no effect upon hMAD2 interaction.

with highest levels in skeletal muscle followed by heart > placenta, brain > pancreas > kidney > lung, liver. To begin to delineate the domain of hMAD2, which was necessary for interaction with the IR, we generated three hybrid proteins and analyzed their interaction in the two-hybrid assay. As shown in Fig. 1C, neither the amino-terminal 136 amino acids nor the carboxyl-terminal 161 amino acids showed any interaction with the IR. This suggested that an intact hMAD2 protein is required for interaction with the IR and/or for proper folding of hMAD2.

hMAD2 Interacts with the COOH Terminus of the IR in a Kinase-independent Manner but Not with the IGFIR—we tested the interaction of hMAD2 with the intact IR hybrid as well as with a kinase-inactive IR in which the critical lysine within the ATP-binding domain (K1018) was changed to alanine. Surprisingly, we observed significant activity in both the Leu prototrophy assay (Fig. 2B) and in the solution assay of β-galactosidase activity (Fig. 2C). This suggested that the interaction of hMAD2 with the IR did not require tyrosine kinase activity. We next analyzed whether or not the full-length hMAD2 hybrid could interact with the related IGFIR in the two-hybrid assay. As shown in Fig. 2 (B and C), no interaction was observed with the IGFIR either in the colony growth assay or in the solution assay of β-galactosidase activity. This finding allowed us to test interaction of hMAD2 with a chimeric receptor in which the COOH-terminal 107 amino acids of the IR were fused to the remainder of the IGFIR cytoplasmic domain (33). As shown in Fig. 2 (B and C), this chimeric receptor was able to interact with hMAD2. In addition, a truncated IR in which the COOH-terminal 30 amino acids (Δ30) have been removed due to insertion of a stop codon was unable to interact with hMAD2. We conclude from these studies that the COOH-terminal 30 amino acids of the IR are required for hMAD2 interaction. Because the COOH terminus of the IR contains two tyrosine residues that are phosphorylated after IR activation, we were interested to determine whether mutation of these tyrosines would have any effect upon hMAD2 interaction. We therefore mutated these residues to phenylalanine either singly or doubly. As shown in Fig. 2 (B and C), these mutations had no effect upon hMAD2 interaction.

In Vitro Interaction of hMAD2 and the IR—To further test the interaction between the IR and hMAD2, we produced a hMAD2-GST fusion protein using amino acids 13–205 of hMAD2. We utilized this fusion protein in GST “pull-down” assays in which we added the immobilized GST fusions to lysates from CHO-IR cells (39) (which overexpress the IR) that were either unstimulated or stimulated with insulin to activate the IR. To assess interaction with the IR in vitro, we analyzed proteins that coprecipitated with these GST fusions by subsequent immunoblotting with anti-IR or antiphosphotyrosine antibodies. As shown in Fig. 3, the IR was easily coprecipitated with the hMAD2-GST fusion in a hormone-independent manner. This is consistent with our two-hybrid findings, which showed that hMAD2 could interact with the kinase-dead IR hybrid.

We next tested in vitro interaction of the hMAD2-GST protein with the IR that were derived from cells that were stimulated with insulin at concentrations ranging from 0 to 100 nM. As shown in Fig. 4 (top panel), the interaction between the IR and hMAD2-GST showed an insulin concentration-dependent decrease in coprecipitation, suggesting that IR activation by insulin reduces the ability to interact with hMAD2 in vitro. This pattern of an insulin-dependent reduction of hMAD2 interaction was the opposite of that observed with the a Grb10-SH2-GST fusion protein (bottom panel) using the same lysates. Grb10 is an SH2-containing protein that interacts well with the activated IR (26, 42–44) and as predicted, only interacted with...
the IR after tyrosine phosphorylation. This serves as a positive control to show that precipitable phosphorylated receptors were in fact present in the lysates and were available for binding. This suggests that receptors that are fully phosphorylated may be unable to interact efficiently with hMAD2 but have higher affinity for SH2 domains such as that of Grb10.

Several explanations are possible to explain the reduced ability of activated IRs to interact with hMAD2 in vitro. First, as suggested above, the fully autophosphorylated IR may be inaccessible to hMAD2 either due to conformational changes or to direct steric blockade by phosphotyrosine residues. It is also possible that receptor activation in mammalian cells leads to interaction with endogenous proteins, which have a higher affinity for the IR than hMAD2, and thus the hMAD2-GST fusions cannot interact efficiently. To better address these issues, we turned to a second assay of in vitro interaction, which utilized purified IR and hMAD2 proteins. We produced the cytoplasmic domain of the IR and IGFIR as histidine-tagged fusions using a baculoviral expression strategy. These receptors were purified using nickel affinity resins to 90% homogeneity as measured by Coomassie staining (not shown). The purified receptors were then stimulated to autophosphorylate in vitro. Unstimulated and stimulated receptors were then electrophoresed using SDS-PAGE and blotted to nitrocellulose. As shown in Fig. 5 (left panels), phosphotyrosine immunoblotting showed that these receptor cytoplasmic domains are efficiently autophosphorylated after the addition of ATP. We next expressed a series of GST proteins that contained a protein kinase A phosphorylation site (40) and could therefore be labeled to high specific activity using purified protein kinase A. We labeled three GST proteins for use as probes: a GST control, labeled to high specific activity using purified protein kinase A, and a series of GST proteins that contained a protein kinase A phosphorylation site (40) and could therefore be labeled to high specific activity using purified protein kinase A. We used this antiserum to immunoprecipitate hMAD2 from CHO-IR cells followed by immunoblotting with antibodies against either the IR or phosphotyrosine. As shown in Fig. 6, we observed immunoprecipitation of the IR with the anti-MAD2 antiserum but not with nonimmune serum. As expected from our previous data, the immunoprecipitation of the IR with hMAD2 was insulin-independent. The phosphotyrosine immunoblot confirms that the coprecipitated protein is in fact the IR because it shows insulin-dependent phosphorylation. These data strongly support the idea that hMAD2 interacts with the IR in cells of mammalian origin.

**DISCUSSION**

Our identification of hMAD2 as a protein that interacts with the COOH terminus of the IR in vitro and in cells is interesting for a number of reasons. First, hMAD2 appears to represent one of the first potential effector proteins that interacts with the IR but not with the related IGFIR. Given the clear physiologically distinct roles that these receptors play, it is possible that hMAD2 may mediate an IR-specific signal. In this regard, however, it is not surprising that hMAD2 interacts with the COOH terminus of the IR because this region shows a high degree of divergence from the IGFIR (47), and this is one of the regions where one would predict receptor-specific proteins would interact. The relatively high level of expression of the hMAD2 mRNA in skeletal muscle at least supports the idea that it may function in IR signaling.

Our findings showing that purified hMAD2 protein can interact with immobilized IR cytoplasmic domains shows unambiguously that this interaction is direct and not mediated by accessory proteins as is possible in the other assays of interaction. This also suggests that the binding domain within the IR is not highly dependent upon the tertiary structure of the IR because the receptor domains were separated using SDS gels prior to blotting to nitrocellulose. Therefore the domain within the IR that interacts with hMAD2 may consist either of a...
simple linear peptide or a more complex domain that is renaturable after blotting and removal of the SDS. Our finding that the autophosphorylated receptor domains produced in baculoviral expression systems show no interaction with hMAD2 in this assay also deserves some discussion. It has been shown that purified receptor kinase domains such as these autophosphorylate very efficiently in vitro, and it is therefore likely that all possible tyrosines are phosphorylated in this system (48). Thus the complete inhibition of hMAD2 interaction is likely to be due to complete autophosphorylation. Conversely, although we observed significantly reduced interaction with autophosphorylated receptors in cell lysates, we never observed complete inhibition, and we did observe coprecipitation of phosphorylated IR from cell lysates (Fig. 3). This is likely to be due to the more heterogeneous nature of the receptors in these systems, some of which may be phosphorylated upon tyrosines that do not inhibit hMAD2 binding. Likewise because we cloned hMAD2 using a kinase-active IR as bait in the two-hybrid assay, it is likely that not all IR baits are fully phosphorylated. We have also carried out experiments to determine whether hMAD2 becomes tyrosine phosphorylated after IR activation. We have been unable to demonstrate any significant tyrosine phosphorylation of hMAD2 in either unstimulated or insulin stimulated CHO-IR lysates (data not shown).

Our demonstration that the COOH-terminal 30 amino acids of the IR are essential for hMAD2 interaction coupled with the apparent inhibition of hMAD2 binding after receptor activation suggests that autophosphorylation of one or both of the tyrosines within this region (1316 and 1322) may serve to inhibit hMAD2 interaction. In this model, IR autophosphorylation would release hMAD2. This might serve to allow hMAD2 to move to its site of action within the cell. Alternatively, this may expose a site upon the IR allowing another effector protein to interact with the IR, and hMAD2 may serve as an inhibitor of signaling prior to receptor activation. It is also possible that receptor autophosphorylation reduces hMAD2 interaction via a structural change within the COOH terminus of the receptor rather than by direct receptor autophosphorylation. Further experiments will be required to characterize the molecular nature of the interaction between hMAD2 and the IR.

Of course the real question that needs to be addressed is the role of hMAD2 in signaling by the IR. We will first discuss what is known about the yeast MAD2 protein. The yeast MAD2 protein was identified as a nonessential gene whose deletion allowed yeast cells to progress through M-phase despite the presence of microtubule polymerization inhibitors such as nocodazole or benomyl (41). Normally cells arrest in mitosis if the chromosomes are not properly attached to the centriole. A number of proteins have been identified in genetic screens as being necessary for this mitotic checkpoint control including three MAD proteins (for mitotic arrest deficient) (41) and three BUB proteins (for budding uninhibited by benomyl) (49). The mechanisms by which these proteins shut down the cell cycle have not yet been clearly delineated. However a model has been proposed that suggests that these proteins may somehow sense an incomplete spindle assembly and inhibit progression through M-phase by inhibition of ubiquitin-mediated proteolysis resulting in the stabilization of proteins such as cyclin B (50). In Xenopus oocytes, it has been proposed that the spindle assembly checkpoint somehow activates ERK2, which is essential for this response. In this study expression of a MAP kinase-specific phosphatase allowed cells to progress through M-phase despite the presence of microtubule polymerization inhibitors (50). Thus one potential role of MAD2 may be to activate the MAP kinase ERK2. In support of the importance of MAD2 proteins in spindle assembly checkpoint control, it has recently been shown that the Xenopus and human MAD2 proteins co-localize with the centromere prior to mitosis, and then during M-phase MAD2 disappears from the centromere (37, 46). Thus MAD2 may interact with incomplete spindle assemblies and be removed after chromosomal attachment. While attached to the centromere, MAD2 may activate the MAP kinase ERK2, thus shutting down the cell cycle until assembly is complete.

Assuming that the only role of hMAD2 is to shut down the cell cycle, it is reasonable to ask what possible role the insulin receptor might play in this. Because the IR is not typically thought of as a stimulator of mitogenesis but rather as a regulator of cellular metabolism, it is possible that the IR might utilize a protein such as hMAD2 to dampen any mitogenic signals emanating from the receptor. In this regard, it has been shown that an IR with a 43-amino acid COOH-terminal deletion shows enhanced mitogenic activity in fibroblasts (51–53). Perhaps this is due to the loss of a signal that normally serves to inhibit cell cycle progression. A similar scenario might be used to explain the enhanced mitogenic signaling by the IGFIR as compared with the IR, because the IGFIR does not interact with hMAD2 and thus may be unable to send out a cell cycle inhibitory signal. The roles of the COOH termini of the IR and IGFIR to signaling remain unclear, although chimeric receptors have suggested that replacement of the IR COOH terminus with that of the IGFIR significantly affected several insulin-stimulated responses (33). Thus hMAD2 may play a role inmediating these responses. Although these are intriguing ideas, if hMAD2 is really only a spindle assemble checkpoint regulator and thus active during late M-phase, there is no a priori reason to implicate growth factor regulation because most growth factors regulate the cell cycle in the G1-phase of the cell cycle, and once S-phase is begun, growth factors are not believed to be necessary for cell cycle progression. It therefore seems unlikely that insulin would play a role in the regulation of mitotic checkpoint control. Therefore, with the exception of a possible role for hMAD2 in the activation of MAP kinases, there is no obvious mechanism by which the insulin receptor might signal via the hMAD2 protein. Of course it remains possible that the hMAD2 protein plays a different role in insulin signaling. Further work is in progress to assess the functional role of the hMAD2 protein in IR signaling.

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