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Two New Substrates in Insulin Signaling, IRS5/DOK4 and IRS6/DOK5

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We have identified two new human genes that encode proteins with tandem pleckstrin homology-phosphotyrosine binding (PH-PTB) domains at their amino termini. Because the other known PH-PTB proteins (insulin receptor substrates: IRS-1, IRS-2, IRS-3, and IRS-4, and the downstream of kinases: DOK-1, DOK-2, and DOK-3) are substrates of insulin and insulin-like growth factor (IGF)-1 receptors, we asked whether these new proteins, termed IRS5/DOK4 and IRS6/DOK5, might also have roles in insulin and IGF-1 signaling. Northern analyses indicate that IRS5/DOK4 is ubiquitously expressed but most abundant in kidney and liver. IRS6/DOK5 expression is highest in skeletal muscle. Both proteins are tyrosine-phosphorylated in response to insulin and IGF-1 in transfected cells, although the kinetics differ. Insulin receptor-phosphorylated IRS5/DOK4 associates with RasGAP, Crk, Src, and Fyn, but not phosphatidylinositol 3-kinase p85, Grb2, SHP-2, Nck, or phospholipase Cγ/Src homology 2 domains, and activates MAPK in cells. IRS6/DOK5 neither associates with these Src homology 2 domains nor activates MAPK. IRS5/DOK4 and IRS6/DOK5 represent two new signaling proteins with potential roles in insulin and IGF-1 action.

To accomplish its pivotal role in maintaining in vivo metabolic homeostasis, insulin binding and activates insulin receptors present on insulin-responsive cells. Early cellular events initiated by insulin binding include receptor tyrosine kinase activation and phosphorylation of the insulin receptor substrates (IRSs).1 Phosphorylated IRSs bind and activate SH2 domain enzymes to couple the activated receptors to such downstream metabolic effects as glucose uptake and glycogen and triglyceride synthesis and storage. IRS-1 is the prototype member of the IRS family. Based on shared domain architecture and phosphorylation by insulin and related IGF-1 receptors, the immediate family has been expanded to include three additional IRS proteins (IRS-2, IRS-3, and IRS-4) and three proteins referred to as downstream of kinase (DOK-1, DOK-2, and DOK-3). These seven proteins have similar amino-terminal targeting domains comprising tandem PH and PTB domains and carboxyl-terminal phosphorylation or “activation” domains which, when tyrosine-phosphorylated, dock SH2 domain proteins. The IRS and DOK proteins are expressed differentially in varying tissues and appear to have distinct but potentially overlapping cellular functions.

IRS-1 and IRS-2 are widely expressed, including in tissues thought to be most important for glucose and lipid homeostasis (1, 2). Both proteins are expressed in muscle, liver, fat, and pancreatic islets, although IRS-1 appears to be more important in muscle metabolism, whereas IRS-2 may play greater roles in liver and islet β cells. Phosphorylated IRS-1 and IRS-2 both bind and activate the SH2 domain proteins PI 3-kinase, SHP2, and Grb2, although PI 3-kinase activation appears to be most important in insulin-mediated glucose homeostasis. IRS-1−/− mice are small and insulin-resistant (predominantly in muscle) but in general do not develop diabetes (3, 4). IRS2−/− mice develop diabetes due to combined insulin resistance (predominantly in liver) and a diminished insulin secretory capacity (5); the females are infertile (6). IRS-3 expression in rodents is restricted primarily to fat, where it binds and activates PI 3-kinase and SHP2 (7). IRS3−/− mice appear normal. IRS-3 has not been identified in the human genome. IRS-4 is expressed predominantly in brain and thymus, where it may bind PI 3-kinase and Grb2. IRS4−/− mice appear normal with the exception of reduced fertility (8).

Although the DOK proteins have similar domain architectures, they can be distinguished from the IRS family based on sequence homology (see below) and functional interactions. DOK-1 is phosphorylated prominently in v-Src, v-Abl, and v-Fps transformed cells and in response to receptor tyrosine kinase activation (9–12). These kinases are different from those typically associated with the IRS activation. DOK-1 was discovered through its association with RasGAP, an SH2 domain-containing protein that does not associate significantly with the IRSs and appears to interfere with MAP kinase activation downstream from B cell and FcγRIIb receptor activation (13). Less is known about DOK-2 and DOK-3, although these too appear to associate with RasGAP and Nck (14–18). All three DOK proteins appear to have functions in lymphocytes and myeloid cells. Targeted deletion of DOK-1 in mice has no overt phenotype, possibly due to a compensatory effect of DOK-2 or DOK-3 (13). Knockouts of DOK-2 or DOK-3 have not been reported. We have identified two additional members of the IRS/DOK family in the human genome data base, based on their having amino-terminal PH and PTB domains, and we characterized these proteins in terms of potential functions in...
insulin and IGF-1 signaling. The recently reported DOK-4 and DOK-5 proteins may be the mouse orthologs (19), although expression patterns and potential biological functions appear to be distinct.

MATERIALS AND METHODS
cDNA Isolation and Plasmid Construction—Full-length cDNAs encoding the human proteins were amplified from a skeletal muscle cDNA library (Clontech) by PCR methods using primers 5'-CCGGAATTCT-GGCCGACCAATTTCTAGTGAC-3' and 5'-CCGCTCGAGTCATGCTGCA-GTGATTCTTGCT-3' (for IRS5/DOK4) and 5'-CCGGAATTCTGGGCTCC-AATTTAATGACATG-3' and 5'-CCGCTCGAGTCATGTCGATCTCTACGAT-CCTTACG-3' (for IRS6/DOK5). EcoRI and XhoI restriction sites were incorporated at the 5' ends. PCR products were purified by agarose gel electrophoresis and sequenced. Vectors for expression of FLAG-tagged proteins in eukaryotic cells were generated by inserting the IRS5/DOK4 and IRS6/DOK5 cDNAs into pCMV-Tag2 (Stratagene).

Northern Blot Analyses—IRS5/DOK4 and IRS6/DOK5 cDNAs were used as probes for Northern blot analyses. cDNAs were excised from the cloning vector, labeled with [α-32P]dCTP (PerkinElmer Life Sciences) by the random hexamer method (Invitrogen), and purified by PCR purification (Qiagen). Human multiple tissue Northern blots were purchased from Clontech. Filters were serially hybridized with a human IRS5/DOK4 and IRS6/DOK5 probes using ExpressHyb hybridization solution (Clontech) and washed twice with 0.2% SSC containing 0.1% SDS at room temperature for 20 min and twice with 0.1x SSC containing 0.1% SDS at 55 °C for 20 min, and exposed to x-ray film overnight at ~ 80 °C.

Cell Culture and Transfections—CHO-IR (20) and CHO-IGF1R (21) cells were maintained in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma) in the presence of 0.4 mg/ml G-418 and 2 μg/ml thymidine under 5% CO2. Cells at 50–60% confluence were transfected with pCMV(IRS5/DOK4), pCMV(IRS6/DOK5), pCMV-1R, or cDNA encoding DOK-1, DOK-2, and DOK-3 (Fig. 1). Membranes were washed twice with 1x SSC. Membranes were incubated 4h at 4°C with immobilized GST-SH2 fusion proteins were analyzed on a SDS-PAGE by Coomassie Blue staining. Equivalent amounts of the immobilized GST-SH2 fusion proteins were incubated for 4 h at 4 °C with cell lysates prepared from transfected, insulin-stimulated (10−8 M, 15 min) CHO-IR cells. Proteins were eluted from the washed beads with Laemml sample buffer, separated by SDS-PAGE, and detected by immunoblotting.

RESULTS
Domain Architecture and Structural Homology—The human genome data base was searched for genes encoding new PH-PTB domain proteins with potential roles in receptor tyrosine kinase signaling. Two genes, located on chromosomes 16q13/21 and 20, were identified. The encoded proteins contain 326 and 306 residues, respectively, and have predicted molecular masses of 37.1 and 35.5 kDa (Fig. 1A). Both of the proteins have amino-terminal PH and PTB domains and short carboxy-terminal tails containing a few tyrosine motifs. Because all known proteins with tandem PH and PTB domains have been categorized either as IRS or DOK proteins, we wondered whether these new members of the family functioned downstream from insulin receptors and whether they were more related to IRS or DOK proteins.

The PH domain of the larger protein, referred to as IRS5/ DOK4, shares 22% identity with IRS-1 and IRS-2, 15–19% identity with IRS-3 and IRS-4, and 16–21% identity with DOK-1, DOK-2, and DOK-3 (Fig. 1B). The PH domain of the smaller protein, IRS6/DOK5, shares 20–25% identity with IRSs and 14–23% identity with DOKs. These levels of PH domain identity are lower than those shared by the IRS (33–58%) or DOK (40–45%) proteins within their own families (Fig. 1B). Because the PH domains of IRS5/DOK4 and IRS6/DOK5 are 60% identical, we conclude that on the basis of PH domain identity, these two newly identified proteins are more closely related to one another than they are to either the IRS or DOK families.

Similar comparisons were made between PH domain sequences. The IRS5/DOK4 PTB domain shares 20–22% identity with the IRS domains and 30–34% identity with those of the DOKs. The IRS6/DOK5 PTB domain is 21–23% identical with the IRS domains and 31–47% identical to the DOK domains. By contrast, IRS PTB domain sequences are 41–76% identical to each other, and the DOK PTB domain sequences are 43–55% identical. Therefore, based on similarity of PH domain sequence comparisons, the IRS5/DOK4 and IRS6/DOK5 proteins are more related to each other (74% identity) than to the IRS or DOK proteins.

Another short region of homology is shared between IRS5/ DOK4 and IRS6/DOK5. The high identity of the PRSAYWHIT motif (where Y is Tyr286) and PRSAYWHQHT motif (where Y is Tyr287) of these proteins suggests a conserved function, possibly binding by Tyr286 or Tyr287, respectively, to SH2 domain proteins. Each protein contains additional tyrosines outside of this shared motif. IRS5/DOK4 has a total of five tyrosines outside of its PH-PTB targeting region within its putative carboxyl-terminal phosphorylation domain: Tyr267, Tyr259, Tyr257, Tyr286, and Tyr288 (Fig. 2). The IRS5/DOK4 and IRS6/DOK5 probes were more related to each other (74% identity) than to the IRS or DOK proteins.

Northern Analyses of IRS5/DOK4 and IRS6/DOK5 Expression—Patterns of gene expression were explored using the full-length cDNAs to probe multiple human tissue mRNA blots (Fig. 2). The IRS5/DOK4 and IRS6/DOK5 probes hybridized...
transcripts of ~3.0 and 2.2 kb, respectively (Fig. 2). IRS5/DOK4 expression was greatest in kidney and liver, with low levels of expression in essentially all other tissues. IRS6/DOK5 expression was very high in muscle, lower in brain, heart, and kidney, and virtually undetectable in other tissues. These are interesting expression patterns in terms of insulin action as muscle and liver

![Diagram](image_url)
are two of the most important systemic targets of insulin.

**Insulin-stimulated Tyrosine Phosphorylation of IRS5/DOK4 and IRS6/DOK5**—To be categorized as genuine “IRSs,” the proteins would need to be tyrosine-phosphorylated by IR in addition to having the appropriate PH-PTB domain architecture. Because we have not yet been successful in generating useful anti-IRS5/DOK4 or anti-IRS6/DOK5, we expressed them in HEK293 cells. Cells transfected with pCMV(IRS5/DOK4) or pCMV(IRS6/DOK5) were immunoprecipitated with anti-FLAG antibodies. Peak phosphorylation was similarly studied using CHO-IR cells transfected with pCMV(IRS5/DOK4) or IRS6/DOK5, respectively, as described under “Materials and Methods.”

**Additional studies conducted in CHO-IR cells determined the concentration dependence for insulin phosphorylation.** Cells transfected with pCMV(IRS5/DOK4) or pCMV(IRS6/DOK5) were exposed for 15 min to 0 to 10^{-6} M insulin and lysed, and FLAG-tagged IRS5/DOK4 or IRS6/DOK5 were immunoprecipitated with anti-FLAG antibodies. Western blots developed with anti-phosphotyrosine antibodies revealed significant increases in tyrosine phosphorylation of both proteins, in response to either insulin (Fig. 3A) or IGF-1 (Fig. 3B). Endogenous insulin and IGF-1 receptors in the 293 cells apparently phosphorylate IRS5/DOK4 or IRS6/DOK5.

**Additional studies further evaluated the effects of pervanadate on IRS5/DOK4 and IRS6/DOK5 phosphorylation in comparison with related effects on IRS-1 phosphorylation.** CHO-IR cells expressing IRS5/DOK4, IRS6/DOK5, or IRS-1 were treated with insulin (10^{-7} M, 15 min) and/or pervanadate (1.0 \mu M, 15 min). Phosphorylation of IRS5/DOK4 and IRS-1 was significantly augmented (Fig. 7A), whereas phosphorylation of IRS6/DOK5 was unaffected (Fig. 7B). Therefore, insulin-stimulated tyrosine-phosphorylation of IRS5/DOK4 is countered by a pervanadate-inhibitable phosphatase, analogous to the situation for IRS-1 (Fig. 7A and B). By contrast, insulin-stimulated phosphorylation of IRS6/DOK5 accumulates more slowly (Fig. 5) and is much less prone to dephosphorylation by a pervanadate-inhibitable phosphatase (Fig. 7B).

**IGF-1-Stimulated Tyrosine Phosphorylation of IRS5/DOK4 and IRS6/DOK5**—Another characteristic of the IRS proteins is their capacity to be phosphorylated by the IGF-1 receptor (IGF-1R), which is closely related to IR in terms of primary sequence, three-dimensional structure, and mechanism. We already knew that IGF-1 stimulated the phosphorylation of IRS5/DOK4 or IRS6/DOK5 in HEK293 cells (Fig. 3). To establish further similarities and differences between the IRSs and IRS5/DOK4 or IRS6/DOK5, 1G-1 receptor-expressing CHO cells (CHO-IGF-1R) were transfected with pCMV(IRS5/DOK4) or pCMV(IRS6/DOK5). Amounts of tyrosine-phosphorylated IRS5/DOK4 and IRS6/DOK5 were determined in cells treated
for 0–40 min with 10^{-7} M IGF-1. Phosphorylation of IRS5/ DOK4 increased quickly, within 2 min, reached a maximum within 5–10 min, and dropped again following longer stimulation times (Fig. 8). IRS6/DOK5 phosphorylation occurred rapidly as well and decreased correspondingly at longer stimulation times.

**Fig. 4.** Dose-dependent insulin-stimulated phosphorylation of IRS5/ DOK4 and IRS6/DOK5 in transfected CHO cells. Insulin receptor-expressing CHO-IR cells (20) were transfected with pCMV(IRS5/DOK4) (A) or pCMV(IRS6/ DOK5) (B). Cells were treated for 15 min with varying concentrations of insulin. Proteins in cell lysates were immunoprecipitated (IP) with anti-FLAG or anti-insulin receptor antibodies, separated by SDS-PAGE, and identified by Western blotting (IB) with anti-phosphotyrosine (PY), anti-FLAG, or anti-insulin receptor antibodies.

**Fig. 5.** Time course for IRS5/DOK4 and IRS6/DOK5 phosphorylation. CHO-IR cells transfected with pCMV(IRS5/DOK4) (A) or pCMV(IRS6/ DOK5) (B) were treated for the indicated times with 100 nM insulin. Proteins in cell lysates were immunoprecipitated (IP), separated by SDS-PAGE, and identified by Western blotting (IB). C, quantified data from A.

**Fig. 6.** Effect of pervanadate on IRS5/DOK4 phosphorylation. Serum-starved CHO-IR cells transfected with pCMV(IRS5/DOK4) were treated with 1.0 μM sodium pervanadate, plus or minus 100 nM insulin, for the indicated times. Cells were lysed, and proteins were immunoprecipitated with anti-FLAG antibodies, separated by SDS-PAGE, and identified by Western blotting (IB).

**Fig. 7.** Activation of IRS5/DOK4, IRS6/DOK5, and IRS-1 in transfected CHO-IR cells. CHO-IR cells transfected with pCMV(IRS5/DOK4) (A), pCMV(IRS6/DOK5) (B), or pCMV(IRS-1) (A and B) were treated for 15 min with 1.0 μM sodium pervanadate and 100 nM insulin. Proteins in cell lysates were immunoprecipitated (IP), separated by SDS-PAGE, and identified by Western blotting (IB).
Co-immunoprecipitation of IRS5/DOK4 with Src, Fyn, CrkII, and RasGAP—Subsequent experiments asked whether the SH2 domain pull-down results translated into corresponding interactions in cells. CHO-IR cells were transfected with pCMV(IRS5/DOK4), pCMV(IRS6/DOK5), or salmon sperm DNA (negative control) and were stimulated with or without 100 nM insulin and 1.0 μM sodium pervanadate. Cell lysates were mixed with immobilized GST-SH2 domain fusion proteins for 1 h at 4 °C, and bound proteins were separated by SDS-PAGE and identified by Western blotting with anti-FLAG antibody.

| Protein Domain | IRS5/DOK4 | IRS6/DOK5 |
|----------------|-----------|-----------|
| PI 3-kinase p85 | SH2-SH2   | -         |
| SHP2           | SH2-SH2   | -         |
| RasGAP         | SH2-SH3-SH2 | ++       |
| PLCγ           | SH2-SH2   | -         |
| Grb2           | SH2       | -         |
| Crk            | SH2       | ++        |
| Nck            | SH2       | -         |
| Src             | SH2      | ++        |
| Fyn             | SH2      | ++        |

Participation of IRS5/DOK4 in Insulin-mediated Activation of MAPK—Findings from the GST-SH2 domain pull-down and co-immunoprecipitation experiments prompted a further investigation of potential cellular consequences of SH2 domain protein activation. Pathways leading through RasGAP, Crk, Src, or Fyn could potentially feed into the MAP kinase (MAPK) cascade. We therefore looked at MAPK activation in insulin-stimulated CHO-IR cells that either were or were not transfected with pCMV(IRS5/DOK4). There was a left shift in the insulin dose response of MAPK activation, as well as an increase in its magnitude, in cells transfected with IRS5/DOK4 compared with cells transfected with control DNA (Fig. 10). These consistent findings in multiple experiments further suggested that IRS5/DOK4 may play a relevant role in insulin signaling.

DISCUSSION

Now that all proteins in the human genome with tandem PH-PTB domain architectures are known, we can attempt to subcategorize the two new ones, IRS5/DOK4 and IRS6/DOK5,
as either insulin receptor substrates (IRS) or downstream of kinase (DOK) proteins. The identification of IRS5/DOK4 and IRS6/DOK5 brings the total to nine: four IRS proteins (IRS-1–4), three DOK proteins (DOK-1–3), IRS5/DOK4, and IRS6/DOK5. We seriously doubt that additional genes encoding PH-PTB domain proteins exist in the human or mouse genomes.

Our main reason for being interested in IRS5/DOK4 and IRS6/DOK5 was to determine whether they function, like the IRS and DOK proteins, in insulin action. Although the lack of useful antibodies left us unable to look at the endogenous proteins, Northern analyses showed that the corresponding mRNAs are expressed in relevant and interesting tissues. IRS5/DOK4 message is expressed in highest abundance in kidney and liver. As major sites of in vivo glycogen storage and glucose production, these tissues are responsible for maintaining normal glucose levels during periods of fasting. A diminished capacity of insulin to suppress hepatic glucose production in type 2 diabetes contributes to hyperglycemia. IRS6/DOK5 message is strongly expressed in muscle, with much less expressed in the other human tissues tested. Muscle is the primary site of in vivo glucose disposal. Suppression of insulin-stimulated glucose disposal occurs in insulin resistance and may contribute to hyperglycemia in type 2 diabetes. Liver and muscle and possibly kidney are important potential targets for regulating metabolic homeostasis and important tissues that contribute to the pathogenesis of type 2 diabetes. We were surprised to see that the mRNA expression patterns recently reported in mice (19) are dramatically different from what we had found in humans (see below).

We therefore asked whether IRS5/DOK4 and IRS6/DOK5 are actual insulin receptor substrates. We would have preferred to answer this question by looking at the endogenous proteins in vivo, in relevant insulin-responsive tissues, but useful antibodies do not exist. Therefore, we expressed epitope-tagged versions of IRS5/DOK4 and IRS6/DOK5 in cultured cells. Both IRS5/DOK4 and IRS6/DOK5 were phosphorylated in HEK293 cells that express endogenous insulin and IGF-1 receptors. We also expressed IRS5/DOK4 and IRS6/DOK5 in CHO-IR cells. Due to the robust signals we were able to ask a variety of kinetic and functional questions about these proteins. Although IRS5/DOK4 and IRS6/DOK5 were both tyrosine-phosphorylated in these cells in response to insulin, the kinetics of phosphorylation were dramatically different. IRS5/DOK4 phosphorylation was rapid and robust, and its time course closely matched that of the insulin receptor itself. It rose rapidly within 2 min of insulin treatment and plateaued. By contrast, IRS6/DOK5 phosphorylation was much slower and increased steadily over all times studied (up to 40 min).

To investigate further the relative rates of phosphorylation, and dephosphorylation by relevant protein-tyrosine phosphatases, we treated cells with pervanadate. Pervanadate is an established mimic of insulin action (25), presumably mediated through its actions on cellular protein-tyrosine phosphatases (26). The results were striking in that IRS5/DOK4 phosphorylation no longer plateaued but continued to increase linearly to quite high levels. This is reminiscent of IRS-1 phosphorylation, which also intensifies in the presence of pervanadate, and distinct from IRS6/DOK5 phosphorylation, which was unaffected by pervanadate. Therefore, IRS5/DOK4 behaves more like an IRS in terms of its phosphorylation kinetics.

Our studies with the human proteins suggest that they function in insulin and IGF-1 action, based on tyrosine-phosphorylation by the insulin and IGF-1 receptors. Message for the mouse ortholog of IRS5/DOK4 is expressed widely but at highest levels in lung, heart, and kidney and at a significantly lower level in liver (19). Differences in expression patterns are even more pronounced for IRS6/DOK5 (19). In mice expression is restricted to the brain, but in humans message expression is far more prominent in muscle. Grimm et al. (19) used a complicated strategy to tease out a potential biological function for the mouse proteins in nerve development. Fusing the extracellular epidermal growth factor receptor to an intracellular portion of c-Ret provides an epidermal growth factor-dependent effect on axonal outgrowth (27). When the DOK-4 or DOK-5 sequences, lacking their PH domains, were fused to the carboxyl terminus of the chimera, axonal outgrowth remained (19). These findings were used to argue that DOK-4 and DOK-5 support neuronal differentiation and MAPK activation, although it would be more conservative to conclude that the fused DOK-4 or DOK-5 sequences don’t interfere, i.e. that their presence has no effect on neuronal development.

Human IRS5/DOK4 is potentially interesting in terms of insulin action, as it is rapidly and heavily phosphorylated in response to insulin and, once phosphorylated, binds a set of SH2 domain proteins. Many previous studies (23, 28, 29) have indicated that the metabolic effects of insulin are mediated largely through PI 3-kinase activation. This is accomplished by the engagement of PI 3-kinase p85 SH2 domains with phosphorylated YXXM and YXXX motifs of the IRS proteins. Neither IRS5/DOK4 nor IRS6/DOK5 contain these sequences, and they do not appear to activate PI 3-kinase directly. In fact, because IRS5/DOK4 and IRS6/DOK5 are significantly shorter than other IRS and DOK proteins, they contain fewer potential sites for tyrosine phosphorylation: five in IRS5/DOK4 and only three in IRS6/DOK5 (Fig. 1A). Screens of a variety of SH2 domain proteins revealed a subset that bound insulin-activated IRS5/DOK4 but not IRS6/DOK5, including RasGAP, Crk, and the non-receptor tyrosine kinases Src and Fyn. The SH2 domains of RasGAP couple Ras to its GTPase activity, which returns GTP-bound Ras to its inactive, GDP-bound state. Because the DOK proteins were originally identified as RasGAP-binding proteins (10, 11), this would suggest from a functional sense that IRS5/DOK4 may be more similar to the DOKs. Crk is an adapter protein that lacks intrinsic catalytic activity but couples tyrosine-phosphorylated proteins to guanine nucleotide exchange factors of Ras-like proteins. RasGAP and Crk and the Src family non-receptor tyrosine kinases (30) all have the potential to influence MAPK/extracellular signal-regulated protein kinase signaling cascades.

PI 3-kinase and MAPK activation represent the two main arms of “classical” insulin receptor signaling cascades. Because IRS5/DOK4 and IRS6/DOK5 have no apparent role in PI 3-kinase activation (data not shown), we investigated the possibility of their inhibiting or activating MAPK, predicting that if enhanced RasGAP activity predominated then MAPK activity would decrease, whereas if Crk/guanine nucleotide exchange factor (31) or Src-like activity predominated then MAPK activity might increase. IRS5/DOK4 expression reproducibly potentiated insulin’s activation of the MAPK. This was evident in both a leftward shift of the insulin dose response and an increase in maximal activation.

We want to emphasize that since our experiments and those of Grimm et al. (19) both utilized overexpression approaches, additional studies will be needed to determine further the functions of endogenous proteins under more physiological conditions. Although our results indicate that IRS5/DOK4 plays a potential role in the mitogenic actions of insulin, relevant animal models will be needed to test this hypothesis. IRS6/DOK5 may play a role in insulin action as well, although discrete mechanisms are less clear.

We were interested in determining whether IRS5/DOK4 and IRS6/DOK5 are members of the IRS family or the DOK family.
of proteins. The answer depends in large measure on how one defines IRS versus DOK families and on whether they represent distinct groups. Historically, the IRSs were named after their roles in insulin receptor signaling (1), whereas the DOKs were named more broadly for roles downstream from any kinase (10, 11). By this definition, the IRSs would be considered to be a subset of the larger DOK family. However, the IRSs were discovered and named long before the DOKs, and based on their being substrates of the insulin receptor, one could readily argue that the DOKs are members of the IRS family (32, 33). We have taken a different approach, which compares protein sequences, particularly in the targeting domains where there is potential for extended homology. IRS-1 and IRS-2 are highly similar; IRS-3 and IRS-4 are more distant relatives, and the IRSs are more similar to one another than they are to other members of either the IRS or the DOK families. Because IRS5/DOK4 and IRS6/DOK5 are much more similar to one another than they are to other members of either the IRS or the DOK families. Because IRS5/DOK4 and IRS6/DOK5 are phosphorylated by the insulin receptor, they are certainly “insulin receptor substrates,” but should they be classified as IRS or DOK family members? We remained sufficiently uncertain that we chose slightly more cumbersome but middle of the road names. Additional studies are clearly needed for a more complete picture to emerge of the biological roles of these interesting proteins in growth, development, and metabolism.

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