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Commentary

Keys to the Hidden Treasures of the Mannose 6-Phosphate/Insulin-Like Growth Factor 2 Receptor

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In 1987, the evidence was published which unequivocally showed that the large mannose 6-phosphate receptor (275 to 300 kd) was also a receptor for the growth factor, insulin-like growth factor II (IGF2).[1–3] Since then, investigation of this multifunctional molecule has continued to reveal interesting information, which extends its relevance across a wide range of biological and pathological processes, from genomic imprinting, human intelligence, to tumor suppression. The paper by Wylie et al in this issue of *The American Journal of Pathology* concerns a conditional Cre/lox disruption of the murine gene, and opens up new and exciting avenues for functional studies in the adult mouse.[4] The purpose of this commentary is to celebrate and reflect on the remarkable progress made during the last decade of study of this receptor, and to highlight future research areas where some questions still remain unanswered.

Both insulin and IGF-I ligands bind receptors that mediate metabolic, growth, survival, and proliferation signals via tyrosine kinase activation. For IGF2, the genetic and biochemical evidence now points toward a receptor, the mannose 6-phosphate (M6P)/IGF2 receptor, as one that binds IGF2 (domain 11) with high affinity, to channel the ligand for degradation within the cell.[5,6] The proliferative and cell survival activity of IGF2 are predominantly mediated via the IGF1 receptor, with contributions from chimeric IGF-1/insulin receptors and isoforms of the insulin receptor.[7]

Gene

The gene coding for the human, bovine, and mouse receptor extends up to 140 kb (human) and comprises a similar number of exons (48).[8,9] Without repeating the Online Mendelian Inheritance in Man (OMIM) database entry (http://www.ncbi.nlm.nih.gov:80/entrez/dispomim.cgi?cmd = entry&id = 147280), there are several important features to point out. First, intron-exon junctions do not appear to map to the 15 extracellular protein domains (~147aa), which all have homology (14 to 28% amino acid sequence identities) to the 7 exon, 159 amino acid extracellular ligand binding domain of the cation-dependent mannose 6-phosphate receptor (CD-MPR). Promoter elements of the receptor have not been fully defined, although there is evidence of four E boxes in the mouse which might bind basic-loop-helix transcription factors such as c-*myc*.[10] An anti-sense transcript is detectable across intron 2 into the promoter on the paternal allele in the mouse. The significance of this relates to the observation that the mouse gene is imprinted. Here, following passage through the parental germline, the maternal-derived allele becomes expressed, but the paternal-derived allele is silenced. The Air (*A*nti-sense *I*gf2r *R*NA) transcript arises from intron 2 and appears to mediate paternal allele gene silencing, that extends over 400 kb to two nearby maternally expressed genes. Deletion of the intron 2 region results in biallelic expression of *Igf2r* when inherited from the paternal allele.[11] Recently, truncation of the Air promoter region or imprinting control center in mouse, resulted in loss of imprinting of *Igf2r* and two of the flanking genes, suggesting that Air has gene repression effects in *cis*.[12] The situation in the human is likely to be different, as both alleles are commonly expressed, and Air RNA is undetectable in first trimester placental tissue.[13–15] In mammalian evolution, data from Jirtle's group[16] has also demonstrated that imprinting of the receptor evolved with the development of the invasive placenta, being absent in birds, but present in marsupials. Interestingly, although *Igf2r* is still imprinted in marsupials, the opossum lacks an intron 2 region of the same sequence as the mouse. This suggests that the silencing of paternal allele expression may either be much more complicated or that mammals and marsupials evolved different mechanisms of imprinting, such as other epigenetic modification, eg, on histones.[17]

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Protein

The M6P/IGF2 receptor is comprised of a 40 residue amino terminal signal sequence, fifteen 124 to 192 amino acid domains, a 23 residue transmembrane domain, and a 167 residue cytoplasmic domain. The protein appears to be expressed ubiquitously, with high expression during development, especially in sites where IGF2 is also expressed. Up to 1.7% of the total protein of the heart is the receptor at day 16 of mouse gestation, with protein levels and mRNA expression falling during the first month of postnatal life. In the human, soluble receptor levels in serum are higher in infants, and also fall in adult life.[18] Most of the protein is detectable within cells around the *trans*-golgi network and endosomal compartment, and a small amount present at the cell surface (5 to 10%), as judged by metabolic labeling and antibody staining.[19,20] Receptors are redistributed to the cell surface following phosphorylation of tyrosine 26, such as occurs after insulin, IGF-II, TGFβ-1 after activation of Type V receptors, and casein kinase II actions.[21] The protein is glycosylated on at least 19 asparagine residues. Recent x-ray crystallographic studies have shed light on the ligand binding domains of the CD-MPR dimer and M6P/IGF2R domain 11, the single IGF2 binding domain of the M6P/IGF2 receptor. In the CD-MPR structure, a flattened barrel structure made of nine strands making two crossed β-pleated sheets form a mannose 6-phosphate binding domain at one pole, with an a helix capping off the base of the barrel at the opposite pole.[22] Disulphide bonds fasten the two β sheets together across a hydrophobic core, with the location of cysteine residues being highly conserved between domains. The CD-MPR molecule forms a dimer with ligand binding sites separate from each other, and the loop that appears to deepen the ligand binding pocket relocates following ligand binding.[22,23] Surprisingly, this structure appears similar to the structure of avidin, which has a deeper ligand binding domain, which may account for the very high affinity of avidin to biotin (10^{-13} M). The structure of the two mannose 6-phosphate receptor binding domains (3 and 9) for M6P/IGF2R has not been solved, but a domain 11 fragment has recently been crystallized.[24] In this case, a similar flattened barrel structure made of two β-pleated sheets forms a shallower IGF2 ligand binding pocket when compared to CD-MPR, and the base of the barrel is capped off by a β-hairpin. Structural predictions suggest that a 43 amino acid region with homology to a fibronectin type II domain might also form a loop that influences IGF2 ligand binding, which is supported by the effects of domain 13 deletion on "off rate" kinetics.[24,25] A further hydrophobic face of the molecule on the external surface of one of the β sheets accounts of the ordered packing within crystals, but also has implications for the organization of the multi-domain protein. This may lead to an organization where even-numbered receptor domains line up on one face of the molecule, opposite the odd-numbered domains that bind the major ligands of mannose 6-phosphate and IGF2.[24] This elongated conformation may agree with previous values of stokes radius.[26] Structural studies so far have not addressed whether the receptor dimerizes at the cell surface. Evidence using chimeric and tagged molecules suggests that receptor dimerization can occur *in vitro*, but it is still unclear as to the demonstration of similar events *in vivo*. The structure of complexes of receptor and ligands have not been reported, although there is potential for mannose 6-phosphate tagged proteins to act as molecular cross-linkers between membrane and soluble forms of the receptor.[26] However, further studies are required to confirm the higher order structure predictions. Finally, a number of other ligands have been shown to bind the receptor, with some effects on cells in culture, but without clear demonstration of effects in the whole organism. These ligands bind via mannose 6-phosphate dependent (latent TGFβ1, proliferin, thyroglobulin, granzyme B, leukemia inhibitory factor)[27,28] and independent mechanisms (retinoic acid, urokinase plasminogen activating receptor).[29]

Function

Early cell culture studies identified the receptor as a functional component of mannose 6-phospate tagged protein transport system (reviewed in[6]). Sorting of acid hydrolases specifically to the pre-lysosomal compartment occurs in the Golgi by the addition of mannose 6-phosphate, binding to either mannose 6-phosphate receptors, budding of clathrin-coated vesicles, and transport to the endosomes (pre-lysosomes). In view of the pH-dependent ligand affinity of mannose 6-phosphate receptors, release of lysosomal enzymes occurs in the acidic pH of the endosomes. Receptors are then recycled, either to the outer cell membrane or return back to the *t*rans-*g*olgi *n*etwork (TGN).[19] Adaptin AP-1 complexes appear to transport from the TGN to endosomes, whereas homologous AP-2 complexes appear to traffic receptors from the cell membranes. Return of receptors from late endosomes to the TGN require signals within the C-terminal tail, and may be regulated through binding of TIP47 and Rab9 GTPase.[30] Furthermore, disruption of the μ1A adaptin gene results in up-regulation of endocytosis internalization rates independent of AP-2, suggesting retrograde AP-1-mediated transport from endosomes to the TGN modify the cell surface cycling of the M6P/IGF2 receptor.[31] Site-directed mutation and chimeric receptors have confirmed the multiple motifs in the cytoplasmic domains that regulate their role in receptor trafficking. At the cell surface, the M6P/IGF2 receptor can bind ligands at neutral pH, in contrast to the CD-MPR which binds at acidic pH, and receptors again appear to cluster at clathrin-coated pits before internalization. Loss of function studies *in vitro* showed that cells that lacked the M6P/IGF2 receptor failed to endocytose the majority of extracellular lysosomal enzymes, an effect that could not be easily compensated by over-expression of CD-MPR. Thus, despite similar trafficking abilities, it appears that the M6P/IGF2 receptor is the main receptor for extracellular ligand interactions.

The first indication that loss of function of the receptor might have dramatic consequences *in vivo* was from the overgrowth phenotype of *Tme* (T-maternal effect) mice.[32]

Gene-specific disruption using homologous recombination in mouse embryonic stem (ES) cells confirmed that disruption of the gene on the maternal allele resulted in disproportional overgrowth, particularly of the heart and placenta, during post-implantation.[33–35] The perinatal lethality was presumed to be due to cardio-respiratory failure. The confirmation that the phenotype was due to unhindered supply of IGF2 derived from the evidence of raised levels of IGF2 peptide and rescue of the phenotype following genetic crosses with *Igf2* knockouts.[33–35] Compensation by the CD-MPR appears to rescue missorting of lysosomal enzymes, which is grossly impaired if both receptors are deficient.[36] Using a constitutive promoter to drive Cre and disrupt M6P/IGF2R, Wylie et al now show the same embryonic overgrowth and lethal phenotype, confirming the effects of loss of function during the embryonic IGF2-dependent growth.[4] However, in view of the lethality, investigation of receptor function in the context of alterations of other ligands has had to await a conditional knock-out as described. As least from initial studies using albumin and creatine kinase promoters, there is little evidence of phenotypic effects after Cre-mediated gene disruption in liver and muscle (cardiac and skeletal), respectively. These results can be explained, as IGF2 ligand supply appears to be critical for embryonic growth before the expression of Cre in these transgenes.[37,38] Thus, the postnatal functions are likely to be unmasked when postembryonic ligands are induced, eg, either from NK T-cell activation for granzyme B, or from reactivation of IGF2 expression in tumors. It should also be remembered that purified soluble forms of the receptor inhibit cell proliferation in culture, perhaps via IGF-II-independent mechanisms.[39] Further, evidence that there might be IGF-II-independent effects of the receptor *in vivo* comes from studies where a soluble form lacking the transmembrane domain was overexpressed in mice using a keratin promoter transgene.[40] Further reduction in growth of the stomach occurred when the transgene was combined with the *Igf2* knockout mouse, suggesting that the receptor may have IGF2-independent effects.[41] Generation of bilallelic expression of the mouse membrane-bound receptor, as is the situation in humans, also results in reduced embryonic growth.[11] Aside from competition between paternal and maternal genomes for the resources extracted from the mother, the so-called "parental conflict" hypothesis, the evolutionary advantage for biallelic receptor expression in humans remains unclear.[42]

A further important functional development has been the identification of loss of heterozygosity (6q27) and associated mutations of the M6P/IGF2 receptor in human cancer. In particular, frequent loss of heterozygosity (LOH) was seen as an early event in the progression of hepatocellular (60%) and breast (30%) tumors, with mutations found also in gastrointestinal and lung cancer.[43–47]A series of careful studies from the Jirtle group have identified a number of frame-shift mutations, missense mutations, and variablity in the size of a polyG tract in exon 28 which leads to protein truncation. The latter mutation was seen relatively frequently in tumors with microsatellite instability, either due to epigenetic silencing or mutation of mismatch repair genes. This mutation along with TGFβ type-II receptor, and others, was used by the National Cancer Institute (NCI) as molecular diagnostic markers of patients with hereditary non-polyposis colonrectal cancer (HNPCC) and associated microsatellite instability.[48] Missense mutations, in particular isoleucine to threonine 1572 common in hepatocellular cancer, abolish IGF-II binding by disrupting the ligand binding pocket in domain 11. This also indicates that the most likely selective pressure within these tumors relates to the supply of IGF2.[49]

Future

The new mouse model might help address a host of basic and medical related questions outlined in their paper; for example, in transplantation, lysosomal metabolism, cardiovascular disease, and intelligence.[4] In particular, this model paves the way for the formal experimental demonstration that the receptor acts as a tumor suppresser gene.

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