Identification of a Novel Ligand-Receptor Pair Constitutively Activated by ras Oncogenes*

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The Ras signaling pathway is thought to control the expression of a subset of yet to be defined genes that are crucial for cell growth and differentiation. Here we have identified by differential display a novel oncogenic Ras target, mob-5, encoding a 23-kDa cytokine-like secreted protein. Mob-5 expression could be induced by oncogenic Ha-ras and Ki-ras, but not by normal ras activation. Inhibitors of both Ha-Ras and mitogen-activated protein kinase kinase completely abolished the mob-5 expression in ras transformed cells, with concomitant loss of the transformation phenotype. Using an alkaline phosphatase-tagged Mob-5 as ligand, a putative Mob-5 receptor was identified on the cell surface of oncogenic ras transformed cells. Thus, the Mob-5/Mob-5 receptor may represent a novel putative autocrine loop coordinately activated by ras oncogenes.

Ras proteins are among the most important molecular switch molecules that relay mitogenic or differentiation signals from the cell surface to the nucleus where selective gene expression takes place. Oncogenic mutations lock the Ras proteins into a permanent “on” position, leading to unregulated cell proliferation, which is the hallmark of cancer (1, 2). Mutations in ras oncogenes have been detected in over 90% of pancreatic cancers and 50% of colorectal cancer (3). In contrast to the extensive body of knowledge related to the genetics of ras activation (4), relatively little is known of the transcriptional events triggered by Ras. Of a few ras target genes previously identified, including transin/stromelysin-1 (5), glucose transporter (6, 7), Pai-2 (8), heparin binding epidermal growth factor (9), and mob-1/IP10 (10, 11), nearly all were shown to be inducible in normal cells by serum growth factors, likely through the activation of endogenous ras (12–14). One long standing puzzle surrounding ras has been that although the effects of Ras activation are very similar to those of serum growth factors, ras-transformed cells may be oncogenic, whereas the parental cells continuously exposed to serum growth factors are not.

To search for additional ras target genes, especially those that may be oncogenic ras-specific, optimized differential display technology (15, 16) was employed to systematically search for such genes in comparative studies involving two carefully chosen paradigms. Since the development of the method, it has been realized that differential display, like other competitive methods such as DNA microarray, is mechanism-based, rather than function-based gene screening tool (17). Therefore, much effort should be made to establish the comparative systems based on as direct and simple a mechanism as possible to identify the relevant genes. To this end, alteration in gene expression in Rat-1 embryo fibroblasts upon conditional expression of oncogenic Ha-ras was analyzed to identify the early ras inducible genes. In the other complementary screening, changes in gene expression was followed in the oncogenic Ha-ras transformed Rat-1 cells upon the treatment with inhibitors of either Ras farnesyltransferase (FTI)1 (18) or MAP kinase kinase (19), which is one of the major Ras downstream kinases required for cell transformation. Here we describe the identification and biochemical characterization of mob-5, the only oncogenic ras-specific gene identified in both paradigms. We demonstrate that mob-5 encodes a cytokine-like secreted protein, which binds specifically to its putative cell surface receptor (Mob-5R) expressed by the ras-transformed cells, but not by their normal parental controls. We propose that the coordinate activation of this novel ligand/receptor loop may play an important role in ras oncogene-mediated neoplasia.

MATERIALS AND METHODS

Cell Lines and Culture—All cell lines including Rat1, Rat1(ras), Rat1-LiRas (10, 35), rat intestinal epithelial (RIE), RIE/Ha-ras, and RIE/Ki-ras (Ko, 1995) were routinely grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% bovine calf serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Life Technologies, Inc.) at 37 °C with 10% CO2. 293T cells and their derivatives were maintained under the same condition as above except 10% fetal bovine serum (HyClone, Logan, UT) was used in place of 10% bovine calf serum. Serum starvation and restimulation of the Rat-1 cells in the absence and presence of cyclohexamide (Sigma) was carried out essentially as described previously (11).

RNA Purification, Automated Differential Display Screening, and Northern Blot Analysis—Total RNA from cells were purified using the RNApure reagent following the manufacturer’s instruction (GenHunter Corp., Nashville, TN). DNase I treatment of RNA prior to differential display was carried out using the MessageClean kit (GenHunter Corp., Nashville, TN). Differential display PCR reactions were setup in 96-well PCR plates (Perkin-Elmer) by the Beckman Biomek 2000 automated liquid dispensing workstation using one-base anchored oligo(dT) primers and rationally designed arbitrary 13-mers (16) from the RNAimage kits (GenHunter Corp., Nashville, TN). The cdnas were amplified in the presence of [a-33P]dATP (NEB Life Science Products) using Taq DNA polymerase (Qiagen) and separated on 6% denaturing polyacrylamide gels (National Diagnostik, Atlanta, GA).

Cloning and Sequencing of the Full-length mob-5 and mda-7 cDNA—Total RNA was isolated from Ha-Ras-transformed rat embryo

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM\EBI Data Bank with accession number(s) AF269251.
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1 The abbreviations used are: FTI, farnesyltransferase inhibitor; MAP, mitogen-activated protein; RIE, rat intestinal epithelial; PCR, polymerase chain reaction; bp, base pairs; IPTG, isopropyl-1-thio-β-D-galactospyranoside; AP, alkaline phosphatase; IL-10, interleukin 10.
fibrolasts as described (16) and then further purified by poly(A) selection using a poly(A)Tract mRNA isolation system (Promega, Madison, WI). The lambda ZAP II Vector/Gigapack cloning kit was used to construct the cDNA library following the instructions provided by the manufacturer. 500,000 plaques were screened for full-length mob-5 cDNA clones. The specific plaques were picked and 384-hp DNA probe from the plaque was labeled with 32P by random primer labeling (GenHunter, Nashville, TN) and the probe was hybridized with the cDNA library. Positive plaques were excised as phagemids and sequenced. After sequencing to ensure the sequence accuracy, the targeting construct was subsequently inserted between the HI-HindIII fragment, containing the ras-5 coding region without its N-terminal 23 signal peptides was generated by PCR using the cloned mob-5 cDNA as a template. The primers used were Lhhis (5'-CCA-GAGCTTCACTGACAGCTCTT-3') and Rhis-mob5 (5'-AGGCTGCTTAGTATCTT-3'). The PCR product was first subcloned into the PCR-TRAP vector (GenHunter, Nashville, TN) and the BamHI-HindIII insert was then excised, purified, and ligated into corresponding sites of the 6×-His tag expression vector pQE32 (Qiagen, Chatsworth, CA). For the expression of recombinant Mda-7–6His-tagged protein, the coding region of Mda-7 excluding the putative signal peptide sequence (codon 1–44) was amplified by PCR using primers Lhis-hMob5 (5'-AGGCTGCTTAGTATCTT-3') and Rhis-mob5 (5'-AGGCTGCTTAGTATCTT-3'). The PCR product was cloned into the PCR-TRAP cloning vector and then excised as a BamHI-HindIII fragment before ligation into competing sites of pQE32. The recombinant plasmids were transformed into Escherichia coli TG1 cells to produce N-terminal 6×-His-tagged Mob-5 and Mda-7. Upon induction with 2 mM IPTG (Life Technologies, Inc.), the recombinant His-tagged Mob-5 and Mda-7 were overproduced as insoluble proteins in the bacteria and purified on a nickel-agarose gel. The amplified mob-5 cDNA was cloned into the PCR-TRAP vector (GenHunter) and completely sequenced.

Expression and Purification of the Recombinant Mob-5 and Mda-7 Proteins and Generation of Polyclonal Antibodies—A 507-bp BamHI-HindIII fragment, containing the rat Mob-5 coding region with its terminal 23 signal peptides was generated by PCR using the cloned Mob-5 cDNA as a template. The primers used were Lhhis (5'-CCA-GAGCTTCACTGACAGCTCTT-3') and Rhis-mob5 (5'-AGGCTGCTTAGTATCTT-3'). The PCR product was first subcloned into the PCR-TRAP vector (GenHunter, Nashville, TN) and the BamHI-HindIII insert was then excised, purified, and ligated into corresponding sites of the 6×-His tag expression vector pQE32 (Qiagen, Chatsworth, CA). For the expression of recombinant Mda-7–6His-tagged protein, the coding region of Mda-7 excluding the putative signal peptide sequence (codon 1–44) was amplified by PCR using primers Lhhis-hMob5 (5'-GGATCCCGATCCGATCC-3') and Rhis-mob5 (5'-GGATCCCGATCCGATCC-3'). The PCR product was cloned into the PCR-TRAP cloning vector and then excised as a BamHI-HindIII fragment before ligation into competing sites of pQE32. The recombinant plasmids were transformed into E. coli TG1 cells to produce N-terminal 6×-His-tagged Mob-5 and Mda-7. Upon induction with 2 mM IPTG (Life Technologies, Inc.), the recombinant His-tagged Mob-5 and Mda-7 were overproduced as insoluble proteins in the bacteria and purified on a nickel-agarose gel. The amplified mob-5 cDNA was cloned into the PCR-TRAP vector (GenHunter) and completely sequenced.

Mob-5 and Mda-7 were used as an intermediate target gene of oncogenic Ha-ras—Using automated differential display screening with rational primer designs (16), two paradigms, one with Rat-LiRas cells containing IPTG inducible oncogenic Ha-ras (9, 11) and the other with oncogenic Ha-ras transformed Rat-1 cells before and after treatment of a MAP kinase inhibitor PD98059 (19), were set up for the systematic screening of oncogenic ras target genes. After screening through 90 combinations of primers representing 70% coverage of the genes expressed in a cell (20), a total of 14 ras inducible genes and 7 ras repressible genes were identified (21). Four of these ras inducible genes, transin/stromelysin-1 (5), osteopontin (22), Cox-2 (23), and Pai-2 (8), were also identified by other methods in previous studies as ras targets. One of the 14 ras inducible genes, designated mob-5, appeared to be novel, and the gene was identified in both screenings using either an inducible ras oncogene or inhibitor, which blocks ras signaling (Fig. 1, A and B). The 394-bp mob-5 cDNA was reamplified from the differential display gels, cloned, and used as a probe to successfully verify the Ha-ras induction of the gene (Fig. 2A). Mob-1, a known ras target gene, previously (10, 11) was used as a control for ras induction. The IPTG treatment of Rat-1:Iras led to the appearance of the oncogenic Ha-Ras protein 4 h post IPTG induction, with concomitant rapid induction of mob-5 mRNA. This result suggests that mob-5 is an early target gene of oncogenic Ha-ras.

Mob-5 Cannot Be Induced by Serum Growth Factors in Nontransformed Rat—Previous studies showed that ras is required for the immediate early response to external growth factor stimulation and normal cell proliferation (12–14). Although mob-5 expression was not detected in nontransformed Rat-1 cells in continuous culture, one would predict that it could be expressed in response to serum stimulation if the gene is a downstream target of the ras signaling pathway. Consistent with this prediction were findings that almost all of the previously identified ras target genes including mob-1 (16), heparin-binding epidermal growth factor (9), Transin (5), osteopontin (22), and glucose transporter (6) could be induced by serum growth factors. To test the serum inducibility of the mob-5 expression, Rat-1 cells were starved for serum and then restimulated with 10% fetal calf serum. Unlike mob-1, mob-5 expression was not detected following serum stimulation, either in the presence or absence of protein synthesis inhibitor (Fig. 2B). In addition, Northern blot analysis failed to detect any mob-5 expression in normal tissues from adult rats, which included brain, heart, lung, kidney, stomach, pancreas, spleen, intestine, skin, skeletal muscle, and blood (data not shown).
Inhibition of mob-5 Expression in Oncogenic Ha-ras Transformed Rat-1 Cells by FTI—The tetrapeptide FTI has been shown to be able to inhibit Ha-ras-mediated cell transformation of rat embryo fibroblasts (18). FTIs inhibit the farnesylation of Ras protein, thereby preventing its membrane localization and biological function. If mob-5 is indeed a target gene of ras, one would predict that FTIs would down-regulate mob-5 expression in oncogenic Ha-ras-transformed cells. To test this, exponentially growing Rat-1(ras) cells were treated with 5 mM of FTI, and the mob-5 mRNA expression and cellular morphology were monitored following the drug addition. As predicted, mob-5 mRNA expression was essentially abolished in 24 h (Fig. 2C), when the cells began to lose the transformed cellular morphology (data not shown).

mob-5 Encodes a Cytokine-like Secreted Protein—Using the 394-bp cDNA probe obtained by differential display, the full-length mob-5 cDNA was isolated from a cDNA library of Ha-ras-transformed rat embryo fibroblasts (10). The 1.2-kilobase mob-5 cDNA encodes an open reading frame of 183 amino acids with an in-frame stop codon 27 bases upstream of the putative translation initiation codon. As predicted, the 394-bp mob-5 cDNA sequence identified by differential display lies at the 3′-end of the mob-5 full-length cDNA. The predicted Mob-5 protein exhibits 68% sequence identity to Mda-7, a gene isolated as a differentiation associated gene from human melanoma cells treated with interferon-β and Mezerein (24 and
The only other gene that showed a significant degree of homology (23% identity) to Mob-5 over most of the coding regions is interleukin 10 (IL-10) from both viral or cellular origins, which is a potent anti-inflammatory cytokine (Fig. 3B and 31). The Mob-5 protein sequence contains a typical signal peptide sequence at its N terminus, suggesting that Mob-5 may be a secreted protein, although previous study failed to demonstrate so for Mda-7 (24).

Overexpression of the full-length mob-5 cDNA with retroviral vector in a variety of rodent fibroblasts, including Rat-1
cells, led to a high level secretion of Mob-5 protein with a predicted molecular mass of 23 kDa. The Mob-5 protein secreted into the culture medium could be readily detected by Western blot using polyclonal antibody directed against the recombinant Mob-5 (Fig. 4A). However, overexpression of Mob-5 alone in Rat-1 cells did not lead to any detectable phenotypic changes, such as morphological transformation.

**Identification of Putative Receptor(s) of mob-5 on the Cell Surface of Ha-ras Transformed Cells**—To determine whether the secreted Mob-5 is a signaling molecule or a cytokine (like IL-10), it is important to know if its specific receptor(s) exist. To this end, APtag technology (26) was employed to create a Mob-5-AP fusion protein, which can then be used as an affinity agent for the receptor analysis. This strategy has been successfully used to identify and clone a number of important cell surface receptors, including those of leptin (27) and semaphorin III (28). Like Mob-5 itself, Mob-5-AP was secreted at a high level from 293T cells stably transfected with the recombinant plasmid. The secreted 90-kDa Mob-5-AP can be readily detected from the culture medium by either the alkaline phosphatase assay or direct Western blot analysis using anti-Mob-5 antibody (Fig. 4A). The construct of the Mob-5-AP fusion protein was also verified by Western blot using antibody to the 67-kDa human placental secreted AP (Fig. 4A, right panel). For the Mob-5 receptor study, Rat-1 cells before and after transformation by oncogenic Ha-ras were incubated with the cell culture medium containing an equal amount of either the Mob-5-AP fusion protein or secreted AP itself. Unlike the AP alone control, Mob-5-AP was found to be preferentially bound to the cell surface of the Ha-ras-transformed cells but not of the parental Rat-1 control (Fig. 4B).

**Activation of mob-5 Expression by Both Oncogenic Ha-ras and Ki-ras in RIE Cells**—Because Ki-ras mutations have been tightly linked with cancers of gastric intestinal origins (3), RIE cells were analyzed for the expression of mob-5. Northern blot analysis was conducted with RIE cells before and after transformation by oncogenic Ha-ras and Ki-ras (29). Consistent with the results obtained with fibroblasts, both oncogenic Ha-ras and Ki-ras were shown to be able to activate mob-5 expression in RIE cells (Fig. 5A).

**Overexpression of mda-7 in Colon Cancer and the Specificity of Mob-5R**—With a high degree of homology to Mda-7, Mob-5 may be the rat homolog of Mda-7. Supporting this hypothesis, mda-7 was found to be overexpressed in nearly all human colon cancer specimens analyzed. However, without any functional data on either protein, one cannot rule out that Mob-5 and Mda-7 are instead closely related members of a same gene family. Based on the published cDNA sequence of mda-7, the entire coding region of the gene was amplified from human colon cancer tissues by reverse transcriptase-PCR. DNA sequence analysis of the resulting mda-7 clone revealed a single amino acid (Ser) insertion between the codon 14 and 15 of the published Mda-7 sequence (24). The Mda-7-AP fusion protein was constructed and expressed in 293T cells. Unlike previous studies on Mda-7, here the Mda-7-AP directed by the signal peptide of Mda-7 was found to be secreted at a high level into the culture medium. The secreted Mda-7-AP could be readily detected by both AP activity assay (range from 0.7 to 1.7 unit/ml) and direct Western blot analysis using polyclonal antibody against Mda-7 (Fig. 5B, left panel). Further confirmation of the 90-kDa Mda-7-AP fusion protein was obtained by Western blot using antibody to AP itself (Fig. 5B, right panel).

As in Rat-1 cells, the putative Mob-5 receptor was also found to be differentially expressed between the normal and Ha-ras-transformed RIE cells using Mob-5-AP fusion protein as a ligand (Fig. 5C, left panel). To determine whether the Mda-7-AP could also preferentially bind to the cell surface of Ha-ras-transformed RIE cells, the receptor binding assay was conducted using Mda-7-AP in place of Mob-5-AP. Compared with the AP control, Mda-7-AP did not exhibit any preferential cell surface binding to the RIE cells before and after transformation by Ha-ras oncogene (Fig. 5C, right panel).

**Regulation of Mob-5R by Ras**—To determine if the expression of the putative Mob-5R was indeed regulated by the oncogenic Ras, the cell surface Mob-5R binding activity was carried out, leading to a high level secretion of Mob-5 protein with a predicted molecular mass of 23 kDa.
out for the Rat-1(ras) cells grown for 48 h in the absence or presence of either 10 μM of the MAP kinase kinase inhibitor, PD98059, or 5 μM of FTI. The result showed that the Mob-5R was dramatically down-regulated by the inhibitor of Ras farnesyltransferase but not that of the MAP kinase kinase (Fig. 6).

**DISCUSSION**

One of the major biological functions of signal transduction pathways is to ensure highly specific control of gene expression in a spatially and temporally regulated manner in response to external stimuli. Although much has been learned in the past two decades about the circuitry of the ras signaling pathway, relative little is known about the downstream genes whose activation at the level of transcription underlies the profound effect on cellular phenotypes during cell growth, differentiation, and transformation. Although differential display and microarray-related technologies have greatly speeded up the identification of differentially expressed genes, finding the true target genes of a given biological system and, in particular, their biological or biochemical functions have been proven extremely challenging. In this study, we described the experimental design that was crucial for the identification of new oncogenic ras target genes by differential display. The criteria were based on 1) activation of expression in normal cells by IPTG inducible oncogenic Ha-ras, 2) down-regulation of expression in ras oncogene-transformed cells by Ras or MAP kinase inhibitors, and 3) the inability to be induced by serum growth factors through normal ras activation. Mob-5 represents the only novel target of ras oncogenes identified by these criteria using primer combinations covering about 70% expressed genes in a cell. The expression of mob-5 could not be detected in nontransformed Rat-1 cells following serum growth factor stimulation.

One of the puzzles surrounding ras has been that although the effects of oncogenic Ras are similar to those of serum growth factors, ras-transformed cells are tumorigenic, whereas the parental cells continuously exposed to serum factors are not. Most of the ras target genes previously identified were shown to be serum inducible, albeit often transiently, consistent with the activation of the normal ras by serum growth factors and its requirement for the G1 cell cycle progression. However, mob-5 expression could not be detected in nontransformed Rat-1 cells following serum growth factor stimulation.
produced by activated T cells, B cells, and monocytes/macrophages (31). IL-10, also known as cytokine synthesis inhibitory factor, functions by binding to its specific cell surface receptor on lymphocytes/macrophages, whereby it elicits a profound anti-inflammatory effect (25). Interestingly, some DNA tumor viruses, such as the Epstein-Barr virus, encode its own vIL-10 gene, which has been shown to be important in tumorigenesis (32). In light of this, we hypothesize that Mob-5 may participate in ras-mediated tumorigenesis by working in an autocrine or paracrine fashion. This hypothesis is supported by our finding that the expression of mob-5 and its putative receptor are coordinately activated by the oncogenic ras in both Rat-1 and RIE cells. However, it is interesting to note that whereas both Mob-5 and Mob-5R are activated by the ras oncogenes, the signaling pathway downstream of Ras appears to have diverged, with Mob-5 expression being dependent and Mob-5R expression being independent of MAP kinase pathway.

Contrary to the previously published results, this study also demonstrates that both Mda-7 and Mda-7-AP fusion protein are secreted. However, unlike Mob-5-AP, Mda-7-AP fails to bind specifically to the cell surface of Ha-ras-transformed RIE cells. It is possible that, like some of the cytokine receptors including that of IL-10, Mob-5R may be species specific (33). Alternatively, Mob-5 and Mda-7 may not be homologs but rather closely related members of the same gene family. Future functional studies on Mob-5 and Mda-7 will help to distinguish the two possibilities. Nonetheless, this finding provides another important experimental control, which supports that Mob-5 binding to ras-transformed cells is receptor-mediated. It should be pointed out that Mda-7, which we cloned from human colon cancer tissues, has a single amino acid insertion (serine 15) in the signal peptide region of the published Mda-7 sequence. It is possible that this difference could explain the discrepancy between the current and previous studies on Mda-7 with regard to its ability to be secreted by the cell.

Although mda-7 was identified as a gene induced by the simultaneous treatment of IFN-β and Mezerein to certain melanoma cell lines as they undergo terminal cell differentiation (24), the molecular mechanism underlying the mda-7 activation and its role in cell differentiation remain obscure. Ectopic expression of mda-7 in some melanoma or other types of cancer cell lines appeared to cause marginal growth inhibition (24, 30). But conflicting data were shown that mda-7 was constitutively expressed in several melanoma cell lines (including SV40-transformed cells) even in the absence of IFN-β and Mezerein treatment (24). It should be interesting to find out whether the apparent cell growth inhibition by Mda-7 overexpression is mediated through the receptor binding, if so, one would predict that Mda-7 added to the culture medium should have a similar effect. Our findings that mob-5 and its putative receptor were specifically activated by ras oncogenes in both fibroblasts and intestinal epithelial cells and that Mda-7 was overexpressed in colorectal cancer seem to contradict the proposed tumor-suppressing function of mda-7 (30). While this work was in progress, a gene called C49a was identified also by differential display to be transiently induced during wound repair in a rat model and the expression of the encoding protein was localized to fibroblast-like cells at the wound edge (34). Interestingly this gene is essentially identical to mob-5. Thus, mob-5 may be involved in pathological processes such as wound repair or inflammation. Thus, this fits well into the notion that “cancer is a wound that never heals.” The constitutive activation of mob-5/mda-7 and their receptors by ras oncogene may offer a potential molecular basis for such an analogy. Conceivably, Mob-5/Mda-7, when constitutively activated by the ras oncogene, could function either in an autocrine fashion to pro-
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mote cancer cell growth or/and in a paracrine fashion as a cytokine to modulate host immune surveillance. Our ongoing effort in functional studies suggest that Mob-5 is distinct from IL-10 based on the inability of Mob-5/MDA-7 either to bind to IL-10 receptor or to inhibit cytokine synthesis of peripheral blood mononuclear cells stimulated with lipopolysaccharide. The molecular cloning of the Mob-5/MDA-7 receptors using the Mob-5-AP/MDA-7-AP fusion proteins should enable us to further establish the functional role of this novel putative ligand-receptor pair in ras-mediated oncogenesis.

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