Contact Inhibition of Hepatocyte Growth Regulated by Functional Association of the c-Met/Hepatocyte Growth Factor Receptor and LAR Protein-tyrosine Phosphatase*

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Contact inhibition, the inhibition of cell proliferation by tight cell-cell contact is a fundamental characteristic of normal cells. Using primary cultured hepatocytes, we investigated the mechanisms of contact inhibition that decrease the mitogenic activity of hepatocyte growth factor (HGF), focusing on the regulation of c-Met/HGF-receptor activation. In hepatocytes cultured at a sparse cell density, HGF stimulation induced prolonged c-Met tyrosine phosphorylation for over 5 h and a marked mitogenic response. In contrast, HGF stimulation induced transient c-Met tyrosine phosphorylation in <3 h and failed to induce mitogenic response in hepatocytes cultured at a confluent cell density. Treatment of the confluent cells with HGF plus orthovanadate, a broad spectrum protein-tyrosine phosphatase inhibitor, however, prolonged c-Met tyrosine phosphorylation for over 5 h and permitted the subsequent mitogenic response. The mitogenic response to HGF was associated with the duration of c-Met tyrosine phosphorylation even in the sparse cells. We found that the activity and expression of the protein-tyrosine phosphatase LAR increased following HGF stimulation specifically in confluent hepatocytes and not in sparse hepatocytes. LAR and c-Met were associated, and purified LAR dephosphorylated tyrosine-phosphorylated c-Met in vitro phosphatase reactions. Furthermore, antisense oligonucleotides specific for LAR mRNA suppressed the expression of LAR, allowed prolonged c-Met tyrosine phosphorylation, and led to acquisition of a mitogenic response in hepatocytes even under the confluent condition. Thus functional association of LAR and c-Met underlies the inhibition of c-Met-mediated mitogenic signaling through the dephosphorylation of c-Met, which specifically occurs under the confluent condition.

Although contact inhibition is a general characteristic of normal cells, the extent to which cells are inhibited by cell-cell contact differs significantly depending on cell types. Hepatocytes in primary culture provide a most appropriate experimental model in investigating contact inhibition (6–9). Mature hepatocytes, which are terminally differentiated cells, undergo proliferation depending on exogenous growth factors. Proliferation of primary cultured hepatocytes is strictly regulated by cell-cell contact. When hepatocytes are cultured at sparse cell density, the cells undergo DNA synthesis in response to growth factors. However, when the cells are in a confluent density, DNA synthesis is mostly inhibited even in the presence of growth factors.

Hepatocyte growth factor (HGF)2 plays pivotal roles in hepatocyte proliferation through the specific receptor c-Met tyrosine kinase (10–14). Disruption of the HGF-c-Met pathway in hepatocytes results in impaired liver regeneration (15, 16). c-Met tyrosine phosphorylation is a triggering event that forms phosphotyrosine-mediated activation cascades of the downstream signaling molecules of c-Met to elicit hepatocyte proliferation in response to HGF stimulation (14, 17). Conversely, it has been proposed that protein-tyrosine phosphatase (PTP) activities are involved in regulating the balance of tyrosine phosphorylation states of receptor tyrosine kinases, including c-Met and the possible downstream signal mediators, such as Shc, the Stat families, phospholipase Cγ1, ß-catenin, ë-catenin, and p120-CA in the normal liver (18). However, the precise role of PTPs in hepatocyte proliferation is not well clarified.

In the present study, focusing on the activation states of c-Met, we investigated the mechanisms by which tight cell-cell contact in primary hepatocytes prevents mitogenic response to HGF. We found that LAR, a transmembrane protein-tyrosine phosphatase, plays a definitive role in inactivation, i.e. tyrosine dephosphorylation of c-Met through their physical interaction, which specifically occurs in hepatocytes under the confluent condition. Additionally, specific suppression of LAR expression prolonged tyrosine phosphorylation of c-Met and released contact inhibition. Our results indicate that regulation of the activation states of c-Met by LAR underlies contact inhibition of the mitogenic response to HGF in hepatocytes.

MATERIALS AND METHODS

Cell Culture—Hepatocytes were prepared from 7-week-old male Sprague-Dawley rats by in situ perfusion of the liver with collagenase, as described elsewhere (6, 19). The cells were cultured on plastic dishes coated with type I collagen at a cell density of $1.2 \times 10^7$/cm² for the

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2 The abbreviations used are: HGF, hepatocyte growth factor; LAR, leukocyte common antigen-related protein-tyrosine phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTP, protein-tyrosine phosphatase; DEP-1, high cell density-enriched PTP-1; RT, reverse transcription; ERK1/2, extracellular signal-regulated kinases 1 and 2.
confluent culture and 0.3 × 10^5/cm^2 for the sparse culture. The cells were cultured in Williams’ E medium supplemented with 5% (v/v) fetal bovine serum, 5 nM insulin, and 10 mM dexamethasone under normal atmosphere conditioned by 5% CO_2.

**Analysis of Mitogenic Response**—DNA synthesis of hepatocytes was measured by the incorporation of 5’-bromo-2’-deoxyuridine (BrdUrd) into nuclei, as described elsewhere (20, 21). Briefly, following preculture for 20 h, the hepatocytes were stimulated with 10 ng/ml HGF and labeled with 10 μM BrdUrd for 30 h. The cells were fixed and permeabilized with methanol. The samples were further treated with 2 M HCl for 15 min and neutralized with 0.1 M sodium tetraborate for 10 min. The samples were incubated with anti-BrdUrd mouse monoclonal antibody (BD Biosciences) and probed by Alexa-549-labeled anti-mouse IgG (Invitrogen). The nuclei were stained by Hoechst 33342. The number of the nuclei stained for BrdUrd and Hoechst were counted using laser microscopy.

**Immunoprecipitation and Western Blotting**—For immunoprecipitation, cells were lysed in a buffer composed of 10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 25 μg/ml aprotinin, and the cell debris were removed by centrifugation at 10,000 g for 10 min. The cell lysates were incubated with an anti-c-Met mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-LAR goat polyclonal antibody (Santa Cruz Biotechnology) for 2 h. The immunocomplexes were precipitated with protein A-Sepharose for anti-LAR antibody or protein G-Sepharose for anti-c-Met antibody. The immunoprecipitates or total cell lysates were subjected to SDS-PAGE and the Western blotting. Proteins transferred to polyvinylidene difluoride membranes were incubated with an anti-phosphotyrosine mouse monoclonal antibody (Upstate Biotechnology Inc., Charlottesville, VA), anti-cyclin D1 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-phospho-extracellular signal-regulated kinases (ERK1/2), mouse monoclonal antibody (Cell Signaling Technology, Beverly, MA), anti-ERK1/2 rabbit polyclonal antibody (Upstate Biotechnology Inc.), anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody (Bio-genesis, Poole, Dorset, UK), anti-c-Met antibody, or anti-LAR antibody for 2 h. The membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody, anti-rabbit IgG antibody, or anti-goat antibody (Dako Cytomation, Glostrup, Denmark). The signals were detected with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences).

**In-gel PTP Assays**—PTPs were monitored by an in-gel PTP assay according to the method reported by Burridge and Nelson (22), with some modifications. Briefly, tyrosine residues on poly(Glu:Tyr) peptides were phosphorylated by purified c-Src tyrosine kinase (Promega, Madison, WI) and ATP and used for a substrate of PTPs. The cell membranes and purified LAR were suspended in 50 mM Tris-HCl (pH 7.4), 1 mM Na_3VO_4, and used for the substrate in *in vitro* PTP reactions. For the preparation of LAR, hepatocytes cultured under confluent conditions were stimulated with 10 ng/ml HGF for 5 h. The cells were lysed in a lysis buffer as described above, and LAR was immunoprecipitated from the cell lysates using anti-LAR antibody covalently cross-linked with protein A-Sepharose. The immunocomplexes were washed with 0.5 M NaCl, and LAR was eluted by 100 mM glycine-HCl (pH 3.0). Thereafter, the elution buffer was replaced by the reaction buffer using a Microcon ultrafiltration membrane (Millipore, Billerica, MA). PTP reactions were started by mixing cell membranes and purified LAR, run for 30 min at 30°C, and stopped by the addition of 1 mM orthovanadate. c-Met was immunoprecipitated from the reaction mixtures and then subjected to Western blot analysis to determine the tyrosine phosphorylation and amount of c-Met using anti-phosphotyrosine and anti-c-Met antibodies, respectively.

**Reverse Transcription (RT)-PCR**—Poly(A) RNA was prepared from hepatocytes using a FastTrack poly(A) RNA isolation kit (Invitrogen) and then incubated with DNase to remove any contaminating DNA. First strand cDNA was synthesized by Superscript III reverse transcriptase (Invitrogen) using oligo(dT) primers. The LAR mRNA level was determined by semiquantitative RT-PCR using oligonucleotide primers 5’-CCCTGTCTCCGGAGCTCCTTCTC-3’ and 5’-ACTGTTGACATTCTCCTGCCCATC-3’, respectively, corresponding to nucleotides 3970–3992 and 5202–5227 from the adenine nucleotide of the starting ATG codon of rat LAR mRNA. PCR primers for GAPDH were purchased from TOYOBO (Osaka, Japan). The domain structures of LAR mRNA expressed in hepatocytes were elucidated by RT-PCR for distinct regions of LAR, as illustrated in Fig. 4E. Poly(A) RNA was prepared and reverse-transcribed as described above. PCR was performed using different sets of primers. For Fig. 4E, rows 1–3, forward primer, 5’-ATAGAGGCCGACGGCCGAGTCAC-3’; reverse primer, 5’-TG-GTGTGTCGCTGAGTACCAC-3’; primer 5’-AAGTCTGAGCAGGTGTCAGGCACC-3’; and 5’-TCTCACAGTTGGGTAAGAGACC-3’. For Fig. 4E, rows 4–6, respectively, forward primer, 5’-GGTCCCTTACTCCCGCATGAG-3’; reverse primer, 5’-ACGTCTCCCTGCGGTCGACCAG-3’; 5’-GTAGTAAACCCATTGTTGGTTGC-3’, and 5’-GGAAAGCAGCCGCGCATCCTTCG-3’. For Fig. 4E, rows 7, 8, forward primer, 5’-CTACAGGTTACCTTGTGGC-3’; reverse primer, 5’-CTAGTAAACCTTGGTGGCTG-3’. For Fig. 4E, rows 8–10, forward primer, 5’-GCAAGGCGGGAATGTTA-3’; reverse phosphorylated peptides were stained by Pro-Q Diamond, an in-gel phosphoprotein staining reagent (Molecular Probes, Inc., Eugene, OR). The gel was analyzed using a laser image analyzer, Typhoon (Amersham Biosciences). PTPs were detected as colorless bands.

**Immunostaining for LAR in Hepatocytes**—Hepatocytes were incubated for 5 h with 0.1% paraformaldehyde in phosphate-buffered saline, permeabilized by 0.2% Triton X-100 in phosphate-buffered saline, and incubated with anti-LAR antibody for 30 min. The cells were incubated with Alexa-488-labeled anti-goat IgG. Nuclei were stained with Hoechst 33342. Fluorescent signals for LAR and the nuclei were analyzed by laser microscopy.

**In Vitro PTP Assays**—Hepatocytes cultured under the sparse condition were stimulated by 10 ng/ml HGF for 20 min, harvested, and disrupted in a glass Teflon homogenizer with hypotonic buffer composed of 10 mM HEPES-KOH (pH 7.4), 10 mM MgCl_2, 42 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3VO_4, and 25 μg/ml aprotinin. The nuclei were removed by centrifugation at 600 × g for 5 min, and the supernatants were centrifuged at 10,000 × g for 10 min. The cells (pellet membranes) were suspended in 50 ml of a reaction buffer composed of 60 mM Tris-HCl (pH 7.2), 5 mM EDTA, 10 mM diethiothreitol, and 50 mM NaCl and used for the substrate in *in vitro* PTP reactions. For the preparation of LAR, hepatocytes cultured under confluent conditions were stimulated with 10 ng/ml HGF for 5 h. The cells were lysed in a lysis buffer as described above, and LAR was immunoprecipitated from the cell lysates using anti-LAR antibody covalently cross-linked with protein A-Sepharose. The immunocomplexes were washed with 0.5 M NaCl, and LAR was eluted by 100 mM glycine-HCl (pH 3.0). Thereafter, the elution buffer was replaced by the reaction buffer using a Microcon ultratranfiltration membrane (Millipore, Billerica, MA). PTP reactions were started by mixing cell membranes and purified LAR, run for 30 min at 30°C, and stopped by the addition of 1 mM orthovanadate. c-Met was immunoprecipitated from the reaction mixtures and then subjected to Western blot analysis to determine the tyrosine phosphorylation and amount of c-Met using anti-phosphotyrosine and anti-c-Met antibodies, respectively.
expression of cyclin D1, a G1/S transition marker, were analyzed using agarose gels and visualized by ethidium bromide staining. Antisense DNA to Suppress LAR Expression—Single strand antisense DNA for LAR mRNA was designed as 5'-CATCATGTGATGGTAGCTT-3' corresponding to the 181–201st nucleotide from the starting codon of rat LAR mRNA (Genbank™ accession number L11586), and the control oligonucleotide was designed as 5'-CAGTAGCTGACTTAGCTT-3'. Antisense and control oligonucleotides were added, respectively, to the cultures of confluent hepatocytes using Lipofectamine 2000 reagent (Invitrogen). Twenty hours later, the medium was changed for the fresh one, and the cells were further cultured in the absence or presence of 10 ng/ml HGF for 5 h. Expression of LAR and c-Met and the tyrosine phosphorylation level of c-Met were analyzed by western blotting, as described above. To analyze the effects of the suppression of LAR expression on mitogenic response to HGF, confluent hepatocytes were pretreated with 10 mM antisense or control oligonucleotide for 20 h and subsequently cultured in the presence or absence of 10 ng/ml HGF for 30 h. The mitogenic response in hepatocytes was analyzed by BrdUrd labeling, as described above.

RESULTS
Distinct Mitogenic Response Regulated by Cell-Cell Contact—To demonstrate any cell density-dependent regulation of mitogenic response, primary hepatocytes were cultured at sparse (0.3 × 10^5 cells/cm² at plating) or confluent (1.2 × 10^5 cells/cm² at plating) cell density. Following preculture for 20 h, the cells were cultured in the absence or presence of HGF for 30 h, and S phase DNA synthesis was measured using BrdUrd labeling. Under the sparse condition, only a few cells underwent BrdUrd incorporation in the absence of HGF, and the BrdUrd signals increased following stimulation with HGF (Fig. 1A). The distribution of cells undergoing BrdUrd incorporation indicates that <10% cells entered S phase in the absence of HGF, yet it reached over 76% in the presence of HGF. Under the confluent condition, BrdUrd-positive cells were almost 5% in the absence of HGF. However, in contrast to the sparse conditions, HGF failed to enhance DNA synthesis. The results show that mitogenic responses of hepatocytes are strictly regulated between sparse and confluent conditions. When hepatocytes were cultured at altered cell densities, wherein the cells were confluent or sparsely attached in the same well, HGF markedly stimulated DNA synthesis in the sparse region but did not do so in the confluent region (Fig. 1B), which clearly ruled out the possibility that the contact inhibition may be regulated by humoral factors.

Next, to address whether the distinct mitogenic response of hepatocytes is associated with distinct cell cycle transition, changes in the expression of cyclin D1, a G1/S transition marker, were analyzed using western blots (Fig. 1C). In the sparse hepatocytes, increase in cyclin D1 expression was detected 6 h after HGF stimulation, and it reached a maximal level 12–24 h following the stimulation. In contrast, the cyclin D1 expression did not increase even in the presence of HGF when cells were cultured under the confluent condition. These results indicate that hepatocytes under the confluent condition remain at a quiescent state even after HGF stimulation.

Suppression of Prolonged ERK Activation and c-Met Receptor Tyrosine Phosphorylation under the Confluent Condition—In various types of cells, entry into G1 following extracellular stimuli is regulated by the extracellular signal-regulated kinases ERK1/2 (23). To search for the possible involvement of ERK1/2 in the mitogenic response to HGF in hepatocytes, we first examined the activation of ERK1/2 (Fig. 2A, left panels). The sparse hepatocytes were stimulated with HGF, and the phosphorylation of distinct tyrosine and threonine residues on ERK1/2 were analyzed by Western blotting. The phosphorylation of ERK1/2 was not detectable in the absence of HGF, but it was enhanced at 5 min after the HGF stimulation, which means ERK1/2 were intracellular signal mediators of HGF stimulation. Under the treatment with PD098095, an inhibitor for the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase, an activator of ERK1/2, phosphorylation of ERK1/2, and increase in cyclin D1 expression following HGF stimulation were inhibited. When S phase entry was analyzed by BrdUrd incorporation, treatment with PD098095 abrogated the BrdUrd uptake even after HGF stimulation (Fig. 2A, right graph). This indicates that ERK1/2 activation was essential for the c-Met-mediated G1/S transition of hepatocytes. We next examined whether ERK1/2 activation is affected by cell densities (Fig. 2B). In the sparse cells, ERK1/2 were
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**Figure 2. Changes in ERK1/2 phosphorylation and tyrosine phosphorylation of c-Met following HGF stimulation.** A, involvement of ERK1/2 activation in cyclin D1 expression and DNA synthesis in hepatocytes. Hepatocytes were cultured under the sparse condition in the absence (−) or presence (+) of 10 μM PD90895 and stimulated with 10 ng/ml HGF. Thirty minutes after the stimulation, the cells were harvested, and phosphorylation of ERK1/2 was analyzed by Western blot using anti-phosphorylated ERK1/2 antibody (upper panel) and anti-ERK1/2 antibody (middle panel). Expression of cyclin D1 at 24 h after the stimulation was determined by Western blot (bottom panel). DNA synthesis was measured by BrdU labeling for 30 h (right graph). B, changes in activation of ERK1/2. Hepatocytes were cultured under the sparse or confluent conditions and stimulated with 10 ng/ml HGF. Phosphorylation of ERK1/2 was analyzed using Western blots with anti-phosphorylated ERK1/2 antibody (upper panel) and anti-ERK1/2 antibody (lower panel). C, changes in tyrosine phosphorylation of c-Met within 2 h. D, changes in tyrosine phosphorylation of c-Met until 5 h and the effect of sodium orthovanadate (Na3VO4) on tyrosine phosphorylation of c-Met. Sparse or confluent hepatocytes were stimulated with 10 ng/ml HGF, and c-Met was immunoprecipitated (IP). In B, confluent hepatocytes were cultured in the absence or presence of 1 mM Na3VO4. Tyrosine phosphorylation of c-Met was analyzed by Western blot, using anti-phosphotyrosine (PY) antibody (upper panel) and anti-c-Met antibody (lower panel).

Initially phosphorylated at 5 min and were sustained for up to 3 h after HGF stimulation (Fig. 2B, left panels). In confluent hepatocytes, phosphorylation of ERK1/2 was slightly induced for up to 5 min and was highly enhanced 20 min after HGF stimulation. However, the phosphorylation level gradually decreased and returned to the unstimulated level within 3 h (Fig. 2B, right panels). The results showed that ERK1/2 activation by HGF stimulation was sustained in the sparse cells and transient in confluent cells, demonstrating that intracellular signal transduction leading to ERK1/2 activation was regulated by cell densities.

The difference in the duration of ERK1/2 activation was reminiscent of the possibility that activation of the c-Met/HGF receptor may be initially regulated by cell densities. Thus, we analyzed the tyrosine phosphorylation states of c-Met during HGF stimulation. Hepatocytes cultured under sparse or confluent conditions were stimulated with HGF, and tyrosine phosphorylation of c-Met was analyzed by immunoprecipitation and subsequent Western blot with anti-PY antibody (Fig. 2, C and D). In sparse cells, tyrosine phosphorylation of c-Met was barely detectable in the absence of HGF. On the stimulation with HGF, the tyrosine phosphorylation was induced within 5 min and reached a peak at 60 min after the stimulation (Fig. 2C). Thereafter, the phosphorylation level was sustained for at least 5 h following HGF stimulation (Fig. 2D). In confluent cells, tyrosine phosphorylation of c-Met was similarly induced within 5 min and reached a peak at 20 min following HGF stimulation (Fig. 2C). It was noteworthy that the tyrosine phosphorylation level of c-Met under the confluent condition decreased thereafter, and 3 h after HGF stimulation, it returned to the basal level (Fig. 2D).

Because c-Met tyrosine phosphorylation faded away until 3 h following HGF stimulation in the confluent hepatocytes, we predicted the involvement of tyrosine phosphatase activity in this down-regulation of c-Met phosphorylation. To address this possibility, the effects of orthovanadate (Na3VO4), an inhibitor for protein-tyrosine phosphatasases with broad spectrum, on c-Met tyrosine phosphorylation were analyzed in confluent hepatocytes (Fig. 2D). In the presence of Na3VO4, c-Met tyrosine phosphorylation slightly increased even before HGF stimulation, and the phosphorylation level became progressively enhanced following HGF stimulation. Importantly, even under the confluent condition, the enhanced level of c-Met tyrosine phosphorylation was sustained for at least 5 h by Na3VO4 treatment, indicating that protein-tyrosine phosphatase(s) participates in the loss of sustained tyrosine phosphorylation of c-Met under the confluent condition.

**Prolonged c-Met Activation Is Essential for HGF-mediated Proliferation.—**To determine whether sustained c-Met tyrosine phosphorylation in the presence of Na3VO4 permits cell cycle transition into the S phase, we analyzed the expression of cyclin D1 and BrdUrd incorporation in confluent hepatocytes (Fig. 3, A and B). Consistent with the results shown in Fig. 1, HGF failed to stimulate cyclin D1 expression when cells were cultured at confluent density. However, in the presence of Na3VO4, cyclin D1 expression increased even following HGF stimulation (Fig. 3A). Likewise, in the presence of Na3VO4, 70% of hepatocytes underwent DNA synthesis after HGF stimulation under the confluent condition, whereas HGF or Na3VO4 alone showed no influence on the quiescent states of the cells (Fig. 3B).

Because c-Met tyrosine phosphorylation for <3 h was not associated with mitogenic response, whereas a longer c-Met tyrosine phosphorylation for at least 5 h led to the induction of mitogenic response, sustained tyrosine phosphorylation of c-Met >3 h may be essential to induce the mitogenic response to HGF. To confirm this possibility, sparse hepatocytes were stimulated with HGF for different periods (1, 3, and 5 h), and thereafter, the medium was changed for one free of HGF, and c-Met tyrosine phosphorylation was analyzed (Fig. 3C). Stimulation of sparse hepatocytes with HGF highly induced tyrosine phosphorylation of c-Met within 1 h, whereas the tyrosine phosphorylation returned to the basal level until 2 h after the change of medium. In the course of HGF stimulation for 3 h, c-Met tyrosine phosphorylation was highly detected at 1 and 3 h, but it also returned to the basal level 2 h after the change of the medium. Stimulation of hepatocytes with HGF for 5 h showed sustained c-Met tyrosine phosphorylation during the stimulation. The DNA synthesis for 30 h following the start of HGF stimulation revealed that the limited stimulation with HGF for 1 h failed to induce DNA synthesis, whereas HGF stimulation for 3 and 5 h increased the...
labeling index to 42 and 69%, respectively (Fig. 3D). These results indicate that sustained c-Met tyrosine phosphorylation for over 3 h is required for entry into the S phase.

Expression of LAR in Confluent Hepatocytes—The effects of Na3VO4 on c-Met tyrosine phosphorylation, shown in Fig. 2D, suggested that protein-tyrosine phosphatase(s) responsible for dephosphorylation of c-Met were involved in the dephosphorylation of c-Met. To search for protein-tyrosine phosphatase(s) responsible for dephosphorylation of c-Met, we examined the expression of tyrosine phosphatase(s) in hepatocytes, using in-gel tyrosine phosphatase assays (Fig. 4A). Hepatocytes were cultured under the sparse or confluent conditions, and the extracts prepared from cells following HGF stimulation were subjected to SDS-PAGE using a polyacrylamide gel containing the substrate peptides of tyrosine phosphatases. The dephosphorylated peptides were detected as unstained bands. Four major bands at molecular mass units of 45, 60, 70 (shown in supplemental Fig. S1), and 80 kDa were detected in this assay, and among these bands, the 80-kDa band showed a unique change. The 80-kDa band was not detected in sparse hepatocytes even after HGF stimulation. However, in confluent hepatocytes, the 80-kDa band was detectable at 1 h, and it markedly increased within 3 h following HGF stimulation. The result suggested that the 80-kDa tyrosine phosphatase is a candidate for the protein-tyrosine phosphatase responsible for the dephosphorylation of c-Met under the confluent condition.

Based on the molecular mass, we predicted that the 80-kDa protein-tyrosine phosphatase is a catalytic subunit (P-subunit) of the protein-tyrosine phosphatase LAR. To address this possibility, expression of LAR in hepatocytes was analyzed by Western blot using anti-LAR antibody (Fig. 4B). In the sparse cells, expression of LAR was negligible regardless of HGF stimulation. On the other hand, basal expression of LAR was weak but more detectable in the confluent cells, and the expression was markedly stimulated within 3 h after HGF stimulation. Similarly, immunostaining for LAR in hepatocytes revealed that LAR was scarcely expressed in the absence of HGF, and its expression was clearly and specifically enhanced in the dense hepatocytes following 5 h of HGF stimulation (Fig. 4C).

To see whether the increase in LAR protein expression in confluent hepatocytes would be regulated transcriptionally, we measured the expression level of LAR mRNA by RT-PCR (Fig. 4D). Although the levels of RT-PCR products of the LAR mRNA were progressively amplified according to the increase in the reaction cycles, a significant difference in RT-PCR products could not be seen between sparse and confluent cells, and the levels did not increase even after HGF stimulation. The results suggest that the increase in LAR protein following HGF stimulation in confluent hepatocytes is regulated post-transcriptionally.

Because many variant forms of LAR in extracellular and juxtamembrane regions are expressed in a variety of cells and tissues (24–28), we analyzed domain structures of LAR mRNA using RT-PCR, focusing on its extracellular and cytoplasmic juxtamembrane domains (Fig. 4E). Poly(A) RNA was prepared from sparse and confluent hepatocytes, RT-PCR was done using different sets of primers, and DNA sequences of PCR products were determined. The resulting RT-PCR products from confluent hepatocytes are shown in Fig. 4E, and the same pattern was obtained in the case of sparse cells (not shown). Taken together with DNA sequence of RT-PCR products, we found that the sequence of LAR mRNA expressed in the primary hepatocytes was consistent with the sequence of rat LAR identified in the liver (25). LAR expressed in hepatocytes contains eight fibronectin type III domains and is characterized by deletions of amino acid residues WRPEESEDY in the fifth fibronectin type III domain and SSAPSCPNIS in the juxtamembrane domain.
Association of LAR and c-Met in Confluent Hepatocytes and in Vitro Activity of LAR to Tyrosine-phosphorylated c-Met—These expression patterns of LAR are reminiscent of the possible involvement of LAR in the attenuation of c-Met tyrosine phosphorylation in confluent hepatocytes. To assess this possibility, we first analyzed the association of LAR and c-Met. Hepatocytes were cultured again in sparse or confluent conditions, and the cell extracts were subjected to immunoprecipitation and subsequent Western blot analysis (Fig. 5). When c-Met was immunoprecipitated from the sparse cells, LAR was not detected in the immunocomplexes with c-Met (Fig. 5A). In the same experiments for confluent cells, a significant association of LAR and c-Met was detected at 3 and 5 h after stimulation with HGF (Fig. 5A). In the reverse experiments, wherein c-Met was analyzed by Western blotting to the immunoprecipitates with anti-LAR antibody (Fig. 5B), association between LAR and c-Met was confirmed in confluent hepatocytes that were stimulated by HGF over 1 h, whereas LAR was not harvested from sparse cells.

To clarify whether LAR is able to dephosphorylate c-Met, possible catalytic activity of LAR to c-Met was analyzed by in vitro tyrosine phosphatase assays (Fig. 5C). The cell membranes containing tyrosine-phosphorylated c-Met were prepared from HGF-stimulated sparse hepatocytes and were incubated as the substrates with or without purified LAR. Next, the c-Met was immunoprecipitated from the reaction mixtures, and the amount of input c-Met and its tyrosine phosphorylation states were analyzed by Western blot using anti-c-Met antibody (Fig. 5C, middle panel) and anti-PY antibody (Fig. 5C, upper panel), respectively. When LAR was not added to the reaction mixtures, c-Met was detected as a tyrosine-phosphorylated form. However, the c-Met was significantly dephosphorylated when LAR was present in the reaction. Taken together, these results indicate that association between LAR and c-Met facilitates dephosphorylation of the tyrosine residues of c-Met in confluent hepatocytes.

Loss of Contact Inhibition by Specific Suppression of LAR Expression—To obtain further evidence for the functional association between LAR and c-Met, we examined whether the specific disruption of LAR results in abnormality in contact regulation, i.e. acquisition of prolonged tyrosine phosphorylation of c-Met and mitogenic response following HGF stimulation. Hepatocytes under the confluent condition were treated with antisense oligonucleotides for LAR mRNA or control oligonucleotides. An increasing amount of antisense oligonucleotides progressively suppressed LAR-expression, whereas control oligonucleotides showed little effect on expression of LAR in any dosage (Fig. 6A). In hepatocytes treated with control oligonucleotides, LAR-expression was induced (Fig. 6B, upper panel) and c-Met tyrosine phosphorylation was still suppressed at 5 h post-stimulation with HGF (Fig. 6B, middle panel). In contrast, in hepatocytes treated with antisense oligonucleotides, expression of LAR was blocked (Fig. 6B, upper panel), and importantly, tyrosine phosphorylation of c-Met was markedly enhanced following HGF stimulation (Fig. 6B, middle panel).

To determine whether enhanced c-Met tyrosine phosphorylation by disruption of LAR is associated with cell proliferation, confluent hepatocytes were subjected to measurements of DNA synthesis by BrdUrd labeling following antisense or control oligonucleotide treatments (Fig. 6C). In hepatocytes treated with the control oligonucleotides, the labeling index did not increase even after stimulation with HGF. However, in the cells treated with antisense oligonucleotides for LAR, HGF stimulation enhanced the labeling index to a 3-fold higher level than that seen without HGF. These results indicate that specific suppression of LAR expression conferred mitogenic response in hepatocytes even when the cells were at confluent density.
DISCUSSION

LAR is a receptor-like transmembrane protein-tyrosine phosphatase (29). It consists of an extracellular region with three immunoglobulin-like and eight-fibronectin type III structures and a cytoplasmic region containing two tyrosine phosphatase domains (29, 30). The structures of the extracellular region of LAR are motifs characteristic of cell adhesion molecules. These structures imply that LAR participates in the suppression of protein-tyrosine phosphorylation in response to changes in cell-cell adhesion (31). In this study using primary hepatocytes, we found that association of LAR with c-Met is regulated by cell-cell contact and that LAR plays a role in contact inhibition of c-Met-mediated mitogenic response through the dephosphorylation of c-Met, which specifically occurs in the confluent hepatocytes.

We first demonstrated that c-Met tyrosine phosphorylation and ERK1/2 phosphorylation upon HGF stimulation under the sparse condition were sustained much longer than those stimulated under the confluent condition (Fig. 2). Thus, it is considered that earlier inactivation of c-Met and ERK1/2 in the confluent hepatocytes was associated with the loss of mitogenic response to HGF. Previous studies demonstrate that sustained activation of ERK1/2 is required for the enhancement of cyclin D1 expression in the G1 phase in different types of cells (32–35). In the primary hepatocytes, ERK1/2 were dephosphorylated earlier than c-Met in confluent cells (Fig. 2, B and C), suggesting a possible involvement of serine/threonine phosphatase(s) in ERK1/2 inactivation. Nevertheless, the loss of sustained c-Met activation is considered to be a primary event leading to the contact inhibition, because DNA synthesis was not enhanced even in sparse hepatocytes, when c-Met activation was restricted for <3 h by pulse stimulation of HGF (Fig. 3, C and D).

In the present study, we found that expression of LAR was synergically regulated by two conditions (Fig. 4, A–C). The cell-to-cell contact is one of the conditions, because expression of LAR was mostly undetectable in sparse hepatocytes. Previous study has indicated that, in some cancer cell lines, LAR is progressively expressed with increasing cell density (36). The cell density-dependent expression of LAR was mediated by its association with E-cadherin, suggesting that E-cadherin-mediated cell-cell adhesion may be involved in the increase in LAR expression in hepatocytes. Other condition is the stimulation with HGF. The LAR expression was enhanced in confluent cells after HGF stimulation. The increase in LAR expression following HGF stimulation suggests that c-Met-mediated signals are involved in the mechanism for the expression of LAR.

It has been reported that LAR binds to the molecules recruited to cadherin-mediated adherens junctions, such as β-catenin and γ-catenin/plakoglobin (36–39) and LAR dephosphorylates β-catenin (39). These demonstrate that LAR plays a role in cell adhesion-dependent signal transduction by regulating the tyrosine phosphorylation states of the cadherin-catenin complex. Similarly, previous studies reveal that c-Met is associated with E-cadherin (40, 41). Because our present findings showed that LAR was associated with c-Met in confluent but not sparse hepatocytes (Fig. 6), E-cadherin may be involved in a mechanism
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by which tyrosine residues in c-Met are dephosphorylated by LAR at confluent cell density.

One of the extracellular matrix components, laminin-nidogen complex, was identified to be a ligand for LAR (28). However, the laminin-nidogen complex is unlikely to be involved in contact inhibition in hepatocytes, because LAR in hepatocytes lacked insertion in the fifth fibronectin type III domain (Fig. 4E), which is essential for binding to laminin-nidogen complex. As another mechanism for c-Met inactivation, Palka et al. (42) report that the receptor-like protein-tyrosine phosphatase DEP-1 (CD148/PTP-h) forms a complex with c-Met and dephosphorylates tyrosine residues of c-Met. However, we could not detect either expression of DEP-1 or association between DEP-1 and c-Met (data not shown), suggesting that DEP-1 is not involved in the contact regulation, at least in hepatocytes.

In summary, we determined for the first time that activation of c-Met receptor in hepatocytes is tightly regulated by cell-cell contact and that functional interaction between LAR and c-Met is a mechanism accounting for the contact regulation of the mitogenic response in hepatocytes. Our results raise the possibility of involvement of the LAR-c-Met system in the regulation of tissue regeneration and malignant transformation of cells. Further studies on the mechanism for cell contact-dependent regulation of c-Met may shed light on the understanding of the maintenance of tissue architectures.

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