Differential Effects of Leukocyte Common Antigen-related Protein on Biochemical and Biological Activities of RET-MEN2A and RET-MEN2B Mutant Proteins*

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Protein-tyrosine-phosphatases (PTPs), in conjunction with protein-tyrosine kinases, play essential regulatory roles in diverse cellular activities by modulating the phosphorylation state of target proteins. Leukocyte common antigen-related (LAR) protein is a widely expressed receptor-type protein-tyrosine-phosphatase that is implicated in the regulation of intracellular signaling triggered by both cell adhesion and peptide growth factors. The gene for LAR is localized to human chromosome 1p32, a region frequently deleted in tumors of neuroectodermal origin, including neuroblastoma, pheochromocytoma, and medullary thyroid carcinoma. On the other hand, the RET gene codes for a transmembrane tyrosine kinase and is responsible for the development of multiple endocrine neoplasia (MEN) 2A and 2B. To explore the potential role of LAR in RET tyrosine kinase activity and RET-induced signal transduction, we cotransfected LAR and RET with a MEN2A or MEN2B mutation (designated RET-MEN2A or RET-MEN2B) into the NIH 3T3 cell line. Here we show that LAR reduces the constitutive tyrosine autophosphorylation and kinase activity of RET-MEN2A but not RET-MEN2B, accompanying a significant decrease of phosphorylation of phospholipase Cᵦ, AKT, and ERK1/2. Interestingly, LAR expression significantly decreased the levels of disulfide-linked RET-MEN2A dimerization. Moreover, reduced oncogenic activity of RET-MEN2A by overexpression of LAR was observed both by an in vitro colony formation assay and by in vivo tumorigenicity in scid mice. These results thus suggest that LAR may contribute to deactivation of the RET-MEN2A mutant protein and reduction of its oncogenic activity in vivo.

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‡ The abbreviations used are: PTP, protein-tyrosine-phosphatase; RPTP, receptor-type PTP; LAR, leukocyte common antigen-related protein; MEN, multiple endocrine neoplasia; GST, glutathione S-transferase; PTK, protein-tyrosine kinase; P3K, phosphatidylinositol 3-kinase; PLCᵦ, phospholipase Cᵦ; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; CS, C1552S; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SHC, Src homologous and collagen; ERK1/2, extracellular signal-regulated kinases 1 and 2; MBP, myelin basic protein.

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tumors originating from neural crest cells, such as neuroblastoma, pheochromocytoma, and medullary thyroid carcinoma (23–25).

The gene for LAR is localized to human chromosome 1p32, a region frequently deleted in neuroblastoma, pheochromocytoma, and medullary thyroid carcinoma (26, 27), and RET germ-line mutations are responsible for multiple endocrine neoplasia (MEN) 2A and 2B that develop medullary thyroid carcinoma and pheochromocytoma (28). These findings led us to postulate that LAR may modulate the function of the RET-MEN2 proteins in human tumors. In this study, to explore the potential role of LAR for RET tyrosine kinase activities, we cotransfected LAR and RET with a MEN2A or MEN2B mutation into the NIH 3T3 cells. As a result, we found that LAR reduced the levels of phosphorylation, tyrosine kinase activity, and oncogenic activity of RET-MEN2A but not RET-MEN2B. Activation levels of signaling molecules potentially downstream of RET kinase were also down-regulated to variable degrees. These results thus suggested that LAR could play a role in the regulation of the function of RET-MEN2A proteins.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-LAR, anti-PLCγ, and anti-AKT/PKB monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-RET rabbit polyclonal antibody was developed against the C-terminal 19 amino acids of the RET long isoform as described previously (29). Anti-phospho-RET (tyrosine 1062)-specific polyclonal antibody was developed against with a synthetic peptide (IENKLpYGMSDP) corresponding to the residues around tyrosine 1062 of human RET. Anti-phosphotyrosine monoclonal antibody was purchased from Transduction Laboratories (Lexington, MA), and anti-ERK 2 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection—The construction of the Aaptag-1 vector containing a full-length RET cDNA with a MEN2A (Cys-634 → Tyr) or MEN2B (Met-918 → Thr) mutation was described previously (30). pMT LAR plasmid containing a full-length LAR cDNA was provided kindly by Dr. H. Saito (Harvard Medical School, Boston, MA). As a control, a truncated mutant LAR cDNA (LAR-DTP) was generated by ligating a 2638-bp EcoRI/NheI human LAR cDNA fragment, resulting in the generation of a protein of 756 amino acids (Fig. 1). NIH 3T3 cells (5 × 10^6 in a 6-cm-diameter dish) were cotransfected with the designated cell lines, subjected to SDS-PAGE under reducing conditions, and analyzed by immunoblotting with anti-LAR monoclonal antibody. Two protein bands of LAR, a ~200-kDa LAR precursor and a 150-kDa extracellular subunit of LAR, were detected in RET-MEN2A/LAR and RET-MEN2B/LAR cells, whereas cells transfected with LAR-DTP expressed a protein with an apparent molecular mass of ~88 kDa (upper panel). The same filter was stripped and reprobed with anti-RET antibody to show equal expression of the RET proteins in each cell line (lower panel). 175- and 155-kDa RET proteins are shown.

**In Vitro Dephosphorylation of RET Proteins by LAR**—RET proteins were purified from 500 μg of cellular proteins by immunoprecipitation with anti-RET polyclonal antibody. The immunoprecipitated RET proteins were washed three times with the RIPA buffer and twice with the LAR protein-tyrosine-phosphatase (PTP) buffer (25 mM Tris-HCl, pH 7.0, 50 mM NaCl, 2 mM Na3EDTA, 2 mM diithiothreitol, 0.01% Brij-35) and divided into two portions. Then they were suspended in 30 μl of LAR PTP buffer containing 1 unit of purified LAR and incubated for 30 min at 30 °C. The reactions were terminated by adding an equal volume of 2 × SDS sample buffer, boiled for 5 min, and subjected to immunoblotting as described above.

**In Vitro RET Receptor Tyrosine Kinase Assay**—Two hundred micrograms of total proteins was immunoprecipitated with 1 μg of anti-RET polyclonal antibody and incubated with protein A-Sepharose beads. The beads were washed twice with RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate. The lysates were clarified by centrifugation (15,000 × g) for 1 h and incubated with 1–2 μg of primary antibodies for 2 h, and the immunocomplexes were precipitated with protein A- or G-Sepharose beads (Amersham Pharmacia Biotech, Tokyo). Before resolving with different primary antibodies, the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 45 min at 55 °C. Protein concentrations were determined using the Bio-Rad protein assay kit.

**Effects of LAR on Activities of RET-MEN2 Proteins**

Using a chemiluminescence ECL detection kit (Amersham Pharmacia Biotech, Tokyo), the full-length LAR and the truncated LAR mutant LAR-DTP, which generates a protein of 756 amino acids, are shown. The full-length LAR protein is cleaved into two subunits that remain associated by a noncovalent linkage. Immunoprecipitation of the proteins was performed three times with antibody, and activation levels of signaling molecules potentially downstream of RET were also down-regulated to variable degrees. These results thus suggested that LAR could play a role in the regulation of the function of RET-MEN2A proteins.
(Cys-1552 → Ser) mutant has been described previously (32). Escherichia coli NM522 was transformed with the expression plasmids and grown at 24 °C, and protein synthesis was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside. The bacterial pellet was removed by centrifugation, and fusion proteins in the supernatant were bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech), washed three times with 10 volumes of buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 250 mM NaCl), and resuspended in NETN buffer. The resulting cell lysates containing 400 µg of total cellular proteins were incubated for 2 h at 4 °C with GST fusion proteins. The GST protein complexes were washed four times with RIPA buffer, and the beads were resuspended in 30 µl of Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 1.4 mM β-mercaptoethanol, 20 µg/ml bromphenol blue), boiled for 5 min, and then fractionated on SDS-10% polyacrylamide gels. Immunoblotting with anti-RET antibody was performed as described above.

Soft Agar Assay and Tumor Growth in Scid Mice—Soft agar assays were carried out in 6-cm dishes as described by Clark et al. (33). Briefly, 5 × 10^4 cells were suspended in 0.3% agar in DMEM containing 10% CS and 10 mM HEPES (pH 7.4) and added on a bottom layer of 0.6% agar. The plates were incubated at 37 °C in a humidified 7.5% CO2 incubator for 2 weeks. The colonies were also stained with 500 µg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, Deisenhofen) for 2–3 h and counted with a microscope. All assays were done in quadruplicates.

For tumorigenesis studies, 1 × 10^7 cells were washed twice, suspended in 0.2 ml of DMEM, and then inoculated subcutaneously into 10-week-old male scid mice (five mice/group). Local tumor growth was measured daily, and mice were sacrificed 16 days after injection to weigh the tumors.

RESULTS

Expression of LAR, RET-MEN2A, and RET-MEN2B in NIH 3T3 Cells—LAR is initially synthesized as a 200-kDa proprotein that is processed at a pentabasic site by an endogenous protease (a subtilisin-like protease), leading to a complex of two noncovalently associated subunits; the extracellular- or E-subunit (150 kDa) contains the cell adhesion molecule-like domain and the phosphatase or P-subunit (85 kDa) contains an 82-amino acid extracellular region and the transmembrane and cytoplasmic domains (Fig. 1a) (34). After cotransfection of NIH 3T3 cells with RET-MEN2A or RET-MEN2B and LAR or LAR-DTP, stable clones resistant to 250 µg/ml hygromycin B were selected and maintained in DMEM supplemented with 8% bovine calf serum. Immunoblotting of the lysates from cells transfected with the LAR gene demonstrated about 5-fold in-

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**Fig. 2.** Effects of LAR on tyrosine phosphorylation of RET. a, total cell lysates (25 µg of proteins) were prepared from the designated cell lines and resolved by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody (upper panel). The same blot was stripped and immunblotted with anti-RET antibody (lower panel). b, equal proteins (300 µg) from the designated cell lines were immunoprecipitated with anti-RET antibody, followed by immunoblotting with anti-phosphotyrosine (upper panel) or anti-RET antibody (lower panel).

**Fig. 3.** In vitro kinase assay of RET proteins. A, equal amounts of proteins (400 µg) from the designated cell lines were immunoprecipitated with anti-RET antibody, and the immunoprecipitated RET proteins were incubated in the presence of [γ-32P]ATP and myelin basic protein (MBP) and subjected to SDS-PAGE on a 15% gel. Autoradiography of the gel is shown (upper panel). Bands representing autophosphorylated RET proteins and phosphorylated MBP are indicated. Aliquots of the immunoprecipitated RET proteins from each cell line were subjected to immunoblotting with anti-RET antibody to show the presence of equal amounts of RET proteins in the samples (lower panel). B, phosphorylated RET and MBP bands were quantitated by densitometric analysis. Results are plotted as percent decrease in phosphorylation of RET and MBP relative to that in the sample from RET-MEN2A/LAR-DTP-expressing cells. C, MTT assay of RET-MEN2A/LAR-DTP-expressing cells.

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**Table 1.** Summary of effects of LAR on tyrosine phosphorylation of RET. | Condition | RET Phosphorylation | MBP Phosphorylation |
|-----------|-------------------|-------------------|
| RET-MEN2A | 100%              | 100%              |
| RET-MEN2B | 90%               | 90%               |
| RET-LAR   | 80%               | 80%               |
| RET-LARDTP| 70%               | 70%               |

**Table 2.** Summary of in vitro kinase assay of RET proteins. | Condition | RET Phosphorylation |
|-----------|-------------------|
| RET-MEN2A | 100%              |
| RET-MEN2B | 90%               |
| RET-LAR   | 80%               |
| RET-LARDTP| 70%               |

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**Table 3.** Summary of MTT assay of RET-MEN2A/LAR-DTP-expressing cells. | Condition | MTT Activity |
|-----------|-------------|
| RET-MEN2A | 100%        |
| RET-MEN2B | 90%         |
| RET-LAR   | 80%         |
| RET-LARDTP| 70%         |
crease in the abundance of the LAR protein compared with that of the endogenous LAR protein in untransfected cells (Fig. 1b).

The migration of the transfected LAR protein species, a ∼200-kDa precursor and a ∼150-kDa proteolytically cleaved extracellular domain subunit, was detected by anti-LAR monoclonal antibody against the LAR N terminus, whereas cells transfected with the truncated LAR cDNA expressed a protein with an apparent molecular mass of ∼98 kDa (Fig. 1b). However, when the same filter was stripped and reprobed with anti-RET polyclonal antibody, there was no significant difference in the expression levels of the RET proteins in all cell lines examined (Fig. 1b).

**LAR Effectively Dephosphorylates RET-MEN2A and RET-MEN2B**—We first measured the levels of tyrosine phosphorylation and kinase activity of RET in the established cell lines. Equal protein amounts of their lysates (25 μg) were subjected to Western blotting with anti-phosphotyrosine monoclonal antibody, 4G10. As shown in Fig. 2a, the level of tyrosine phosphorylation of the 175-kDa RET in RET-MEN2A/LAR cells was significantly lower than that in RET-MEN2A and RET-MEN2A/LAR-ΔTP cells, whereas there was no difference in the phosphorylation levels of RET among RET-MEN2B, RET-MEN2B/LAR, and RET-MEN2B/LAR-ΔTP cells. Because the decrease of tyrosine phosphorylation of the RET protein was observed in three independent RET-MEN2A/LAR clones (data not shown), we pooled them for the following experiments. In addition, this result was confined by immunoprecipitation of the lysates with anti-RET antibody, followed by immunoblotting with anti-phosphotyrosine antibody (Fig. 2b).

We next measured the RET tyrosine kinase activity in each cell line. Equal amounts (250 μg) of lysates from the cell lines were immunoprecipitated with the anti-RET antibody and incubated in the presence of [γ-32P]ATP and MBP. Phosphorylated products were resolved by SDS-PAGE and detected by autoradiography (Fig. 3a). The relative levels of catalytic activities were calculated by densitometric analyses of each band, and the results were plotted as percent decrease of phosphorylation levels relative to those in RET-MEN2A/LAR-ΔTP-expressing cells (Fig. 3, b and c). We found the RET autophosphorylation and phosphorylation of MBP that was used as an exogenous substrate were reduced by 80 and 70%, respectively, in RET-MEN2A/LAR cells compared with those in RET-MEN2A/LAR-ΔTP cells. Consistent with the previous results (35, 36), the autophosphorylation of RET-MEN2B proteins from RET-MEN2B/LAR and RET-MEN2B/LAR-ΔTP cells was much lower than that of RET-MEN2A proteins. However, the inhibitory effects of LAR on RET-MEN2B autophosphorylation and kinase activity were not observed in RET-MEN2B/LAR cells (Fig. 3). These results confirmed that overexpression of LAR inhibited the tyrosine kinase activity of only RET-MEN2A.

**LAR Effectively Dephosphorylates RET-MEN2A and RET-MEN2B in Vitro**—The above results suggest that RET may be a direct substrate of LAR. To test this possibility, phosphorylated RET was immunoprecipitated and incubated with purified LAR in vitro. As shown in Fig. 4, RET was efficiently and completely dephosphorylated, suggesting that RET is a preferred and efficient substrate of LAR tyrosine phosphatase in vitro.

**LAR Binds to RET-MEN2A and RET-MEN2B in Vitro**—To investigate the mechanism of how LAR inhibits the RET-MEN2A kinase activity and reduces the level of its autophosphorylation, we examined whether LAR interacts with the RET proteins. Initially, cell extracts were incubated with anti-RET polyclonal antibody in the presence of PTP inhibitor (sodium vanadate). Proteins that bound to RET were precipitated with protein A-Sepharose beads and analyzed by immunoblotting with the anti-LAR antibody. However, no LAR was detected by this approach, presumably because active LAR may rapidly dephosphorylate and dissociate the substrate in vivo despite the presence of phosphatase inhibitor (data not shown). To overcome this problem, we used mutant LAR in which Cys-1552 was changed to Ser (C1552S, abbreviated as CS). Because Cys-1552 is located in the catalytic center of LAR, the CS mutant has no phosphatase activity (31), whereas it should retain its affinity for the substrates. A physical association was observed between an analogous CS mutant of the 3CH134PTP and its putative substrate; p42 MAPK (37). Equal protein amounts (250 μg) of the lysates prepared from each transfectant were incubated with GST, GST-LAR, and GST-LAR-CS immobilized on glutathione-Sepharose beads and then separated by SDS-PAGE under reducing conditions. Immunoblot analysis with anti-RET antibody indicated that GST-LAR-CS but not GST-LAR bound to both RET-MEN2A and RET-MEN2B, suggesting that association of wild-type LAR with mutant RET proteins may be transient (Fig. 5).

**Down-regulation of RET-MEN2A-induced Signal Transduction by LAR**—The effects of RET tyrosine kinase are mediated by the concerted activation of several signaling pathways, including SHC, phospholipase Cγ, phosphatidylinositol 3-kinase, and MAPK (38). Thus, we investigated whether all kinase-dependent events are affected to a similar degree by expression of
LAR. To demonstrate these, we have analyzed some occurrences of RET-dependent intracellular signaling in the transfectants. First, equal amounts of the cell lysates (250 μg) were immunoprecipitated with anti-PLCγ antibody followed by immunoblotting with anti-phosphotyrosine antibody. Overexpression of LAR in RET-MEN2A cells caused an ~80% reduction in the tyrosine phosphorylation of PLCγ, compared with that in RET-MEN2A/LAR-ΔTP cells (Fig. 6c). However, the tyrosine phosphorylation levels of PLCγ were approximately equal in RET-MEN2B/LAR and RET-MEN2B/LAR-ΔTP cells. After stripping this membrane, it was reprobed with anti-phospho-PLCγ antibody to verify equal protein loading (Fig. 6a, lower panel).

We also tested the effect of LAR on ERK1/2 and AKT/PKB activation. Cell lysates (25 μg) from each cell line were subjected to SDS-PAGE and analyzed by immunoblotting with anti-phospho-ERK1/2 (Thr-202/Tyr-204) or anti-phospho-AKT (serine 473) antibody. As shown in Fig. 6d, the lysates (25 μg) of the designed cell lines were immunoblotted with anti-phospho-ERK1/2 (tyrosine 1062)-specific antibody (upper panel). The same filter was stripped and reprobed with anti-PLCγ antibody (lower panel). B, the cell lysates (25 μg) were analyzed by immunoblotting with anti-phospho-ERK1/2 (Thr-202/Tyr-204) antibody (upper panel). The same filter was stripped and reprobed with anti-PLCγ antibody (lower panel). C, the lysates (25 μg) were immunoblotted with anti-phospho-AKT (serine 473) antibody (upper panel). D, the lysates (25 μg) from the RET-MEN2A and RET-MEN2A (Y1062F) cells were immunoblotted with anti-phospho-RET (tyrosine 1062)-specific antibody (upper panel). The blots were reprobed with anti-phospho-RET antibody (lower panel). D: the lysates (25 μg) from the RET-MEN2A and RET-MEN2A (Y1062F) cells were immunoblotted with anti-phospho-RET (tyrosine 1062)-specific antibody (upper panel). The blots were reprobed with anti-phospho-RET antibody (lower panel). The same filter was stripped and reprobed with anti-PLCγ antibody (lower panel).

Because ERK1/2 and AKT are known to be activated through phosphorylated tyrosine 1062 in RET (39), we investigated its phosphorylation state using anti-phospho-RET (tyrosine 1062)-specific antibody. To define the specificity of this antibody, we established NIH 3T3 cells expressing RET-MEN2A proteins in which tyrosine 1062 was replaced with phenylalanine (designated Y1062F). As shown in Fig. 6d, anti-phospho-RET (tyrosine 1062)-specific antibody detected tyrosine phosphorylation of RET-MEN2A proteins but not RET-MEN2A (Y1062F) proteins (Fig. 6d, middle panel). As expected, LAR expression significantly reduced tyrosine-1062 phosphorylation in RET-MEN2A but not in RET-MEN2B (Fig. 6e, upper panel). The levels of RET protein expression were comparable in each cell line (Fig. 6, d and e, lower panels).

LAR Inhibits Dimerization and Phosphorylation RET-MEN2A Proteins—The MEN2A mutations activate RET by inducing its ligand-independent dimerization, whereas the MEN2B mutations activate RET without dimerization (30). Thus, we investigated whether LAR expression affects the dimerization of the RET-MEN2A proteins. When the lysates from each transfectant were analyzed by immunoblotting under reducing or nonreducing conditions, LAR expression significantly decreased the dimerization and phosphorylation of RET-MEN2A proteins, demonstrating that their decreased kinase activity correlates with their decreased dimer formation (Fig. 7).

LAR Inhibits RET-MEN2A-mediated Transformation and Soft Agar Growth—We examined whether LAR can reverse cell transformation by RET-MEN2A. RET-MEN2A/LAR-ΔTP cells were rounded, refractile, and highly transformed, whereas the RET-MEN2A/LAR cells resumed a more flattened morphology (Fig. 8, a–c). Morphological reversion of RET-MEN2B/LAR cells was not observed (Fig. 8, d and e). These findings demonstrate that transformation by RET-MEN2A, but not by RET-MEN2B, can be reversed partially by LAR overexpression.

To test possible biological consequences of LAR expression, we performed soft agar colony formation assays. RET-MEN2A/LAR-ΔTP cells displayed typical large colony formation in soft agar (Fig. 8, g and l) similar to that of RET-MEN2A cells (data not shown). Conversely, a small number of colonies of RET-MEN2A/LAR cells were observed under the same conditions, demonstrating that cells expressing LAR, but not LAR-ΔTP, became more anchorage-dependent. Thus, the effect of LAR expression on RET-MEN2A receptor phosphorylation seems to
behave functions as an inhibitor of receptor protein-tyrosine kinase signaling in vivo by direct dephosphorylation of certain growth factor receptors (1), and that LAR could be a tumor suppressor because of its PTP activity, tissue distribution, and chromosome location (43, 44). Because the LAR gene is localized to human chromosome 1p32, which is frequently deleted in tumors developed in MEN2 (26, 27), it was interesting to investigate whether LAR modulates the functions of RET-MEN2 proteins. Thus, we cotransfected LAR or phosphotyrosine domain deletion mutant LAR (LAR-ΔTP) with RET-MEN2A (Cys-634 → Tyr) or RET-MEN2B (Met-918 → Thr) into NIH 3T3 cells, and demonstrated that the constitutive tyrosine autophosphorylation of RET-MEN2A but not RET-MEN2B became much reduced by LAR expression. The in vitro RET-MEN2A tyrosine kinase activity was also markedly decreased (~70–80%). The observations that high levels of LAR protein expression significantly reduced proliferation of RET-MEN2A/LAR cells in vitro as well as tumor growth in vivo clearly emphasize potentially important roles for LAR in the regulation of neoplastic cell proliferation and tumorigenicity. This is the first report, to our knowledge, documenting suppression of biological activities of the RET-MEN2A protein by LAR.

To elucidate the mechanism by which LAR suppresses tumor growth of cells expressing RET-MEN2A, it was essential to investigate the association of LAR with RET-MEN2A. The significant reduction in the proliferation rates of RET-MEN2A/LAR cells probably depends on the LAR function, because cell proliferation is regulated by the phosphotyrosine state in these cells. One possibility is that RET-MEN2A itself is a target substrate for LAR. It has been reported that LAR was able to dephosphorylate autophosphorylated epidermal growth factor receptor in vitro (45) and that LAR and the insulin receptor was able to coinmunoprecipitated after chemical cross-linking on the surface of rat hematoma cells (46). In this report, we have shown that the level of RET-MEN2A tyrosine phosphorylation was significantly reduced by overexpression of LAR although the level of RET expression was unchanged. Like other PTPs, LAR contains a conserved 11-amino acid motif ((IV)HCXAXXR(S/T/G)) within the catalytic domain, which constitutes the active site of the phosphatase (43). The invariant cysteine within this motif is absolutely required for catalysis. N-terminal to this region is a conserved aspartic acid residue (47). A mutation of the cysteine or aspartic acid residue to serine or alanine, respectively, resulted in inactivation of PTPs but the mutant PTPs retained their ability to bind tyrosine-phosphorylated substrates and have successfully been used for the identification of candidate PTP substrates (48). Our results demonstrated that the LAR-CS mutant protein interacts directly with RET-MEN2A as well as RET-MEN2B using the glutathione S-transferase LAR-CS fusion proteins, suggesting that the normal interaction between LAR and RET is likely transient and that RET may be quickly dissociated from LAR after dephosphorylation. Thus, this transient interaction may not have been detected by coinmunoprecipitation even in the vanadate-treated cells (data not shown).

As we reported previously, specific phosphotyrosine residues in RET were required for the mitogenic and transforming activity (29, 49, 50). Upon RET activation, the autophosphorylated tyrosine residues, Tyr-905, Tyr-1015, Tyr-1062, and Tyr-1096, act as docking sites for the signal transduction effectors, Grb7/Grb10, PLCγ, SHC/Enigma, and Grb2, respectively (29, 51–56). This is consistent with the data indicating that several signaling pathways, including phosphatidylinositol 3-kinase (PI3K), RAS/ERK, PLCγ, and c-Jun N-terminal kinase (JNK) pathways are activated by RET. Among these, activation of the RAS/ERK, PI3K/AKT, and JNK signaling pathways depended on phosphorylation of tyrosine 1062 (39, 57). In addition, we demonstrated that a mutation of tyrosine 1062 markedly decreased the transforming activity of both RET-MEN2A and RET-MEN2B (29, 49, 50). The recruitment of the GRB2-SOS

**DISCUSSION**

*In vitro*, it has been proved that intracellular PTPs such as PTP1B and low molecular weight PTP, and transmembrane PTPs such as LAR, RPTP α, and PTP α, have substantial activity toward receptor protein-tyrosine kinases. LAR, RPTP α, and PTP α have been shown to regulate insulin signaling via effects on the activated receptor (40–42). It has been postulated that LAR functions as an inhibitor of receptor protein-tyrosine kinase signaling in vivo by direct dephosphorylation of certain growth factor receptors (1), and that LAR could be a tumor suppressor because of its PTP activity, tissue distribution, and chromosome location (43, 44). Because the LAR gene
complex to SHC bound to tyrosine 1062 was involved in the activation of RAS/ERK signaling pathway, whereas association of SHC with GRB2-associated binder 1 appeared to be essential for the activation of PI3K pathway (39, 58). If RET is the primary target of LAR action, it is expected that the LAR-dependent reduction in its tyrosine kinase activity would be paralleled by reduction in substrate phosphorylation. Consistent with this view, we showed the decreased levels of phosphorylation of AKT, ERK1/2, and PLCγ in the RET-MEN2A/LAR cells. Furthermore, immunoblotting with anti-phospho-RET (tyrosine 1062)-specific antibody revealed the decreased tyrosine-phosphorylation of RET-MEN2A, suggesting that tyrosine 1062, a binding site for SHC adaptor protein, is one of the sites affected by LAR action. Similarly, decreased phosphorylation of PLCγ in the RET-MEN2A/LAR cells suggested that tyrosine 1015 is another target site for LAR.

In this study, another interesting finding is that LAR neither reduces the phosphorylation and the kinase activity of RET-MEN2B nor decreases the tumor growth of RET-MEN2B cells. As we and others reported previously, RET-MEN2A is dimerized through the formation of disulfide bonds between unpaired cysteine residues in the extracellular domains of two molecules, and the levels of its autophosphorylation and tyrosine kinase activity are elevated in parallel (59, 60). On the other hand, the RET-MEN2B mutation does not provoke constitutive dimerization of the RET protein, and activation of RET-MEN2B results from an altered conformation of the kinase domain that appears to lead to altered substrate specificity (30, 49, 58, 60, 61). We therefore hypothesize that the dimerization state of RET-MEN2A may be preferable for LAR action. In addition, we showed that RET dimerization and phosphorylation were significantly reduced in RET-MEN2A/LAR cells as compared with those in RET-MEN2A/LAR-ΔTP cells. This result suggests that LAR expression on the cell surface may interfere with the RET dimerization, resulting in the decrease in its oncogenic activity. In this respect, it is interesting to note that we recently succeeded in generating transgenic mice expressing the RET-MEN2A proteins that showed tissue-specific tumor development. In transgenic mice, no tumor development was observed in several tissues despite the high levels of expression of the RET-MEN2A proteins. We found that RET-MEN2A dimerization was undetectable in these tissues, suggesting that certain cell surface proteins could interfere with the RET dimerization (62). Thus, it is possible that LAR expression plays an inhibitory role in the tumor development in vivo, affecting the RET-MEN2A dimerization as well as its kinase activity, although further analyses are necessary to elucidate the exact mechanism of RET-MEN2A inactivation by LAR.

In summary, this study indicated that LAR specifically suppresses the biological activities of RET-MEN2A by reducing its kinase activity and down-regulating activation of its downstream signaling pathways. Thus, the deletion of the LAR gene that is localized to human chromosome 1p32 may affect biological behaviors of medullary thyroid carcinoma and pheochromocytoma developed in MEN2A patients.

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