The Transmembrane Protein-tyrosine Phosphatase LAR Modulates Signaling by Multiple Receptor Tyrosine Kinases*

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Antisense-mediated suppression of the transmembrane protein-tyrosine phosphatase (PTPase) LAR has been shown previously to increase insulin-dependent phosphatidylinositol 3-kinase (PI 3-kinase) activation by greater than 300% in the rat hepatoma cell line McA-RH7777. Here, insulin-dependent insulin receptor tyrosine kinase activation was examined with recombinant insulin receptor substrate 1 (IRS-1) as the substrate and shown to be 3-fold greater in cells with suppressed LAR levels. Consistent with a receptor level effect, in vivo insulin-dependent tyrosine phosphorylation of both IRS-1 and Shc was increased by a similar 3-fold with LAR suppression. These increases in IRS-1 and Shc phosphorylation were paralleled by increases in insulin-dependent PI 3-kinase association with IRS-1 and activation of the MAP kinase pathway.

Reduced LAR levels also resulted in increases of over 300% and 250% in epidermal growth factor (EGF)- and hepatocyte growth factor (HGF)-dependent receptor autophosphorylation, respectively, as well as a detectable increase in substrate tyrosine phosphorylation. In a post-receptor response, EGF- and HGF-dependent MAP kinase activation was increased by 300% and 350%, respectively, with LAR suppression. Similarly, growth factor-dependent PI 3-kinase activation was increased in LAR antisense expressing cells when compared to null vector expressing cells. These results demonstrate that the transmembrane PTPase LAR modulates ligand-dependent activation of at least three receptor tyrosine kinases.

Although the pathways that become activated by receptor tyrosine kinases have been characterized extensively, the mechanisms by which these receptors can be modulated have not been well studied. A significant number of in vitro studies have demonstrated that protein-tyrosine phosphatases (PTPases) might function as important regulators of receptor tyrosine kinases (reviewed in Ref. 1). We and others have shown that overexpression of PTPases within intact cells results in a blunting of ligand-mediated receptor tyrosine kinase autophosphorylation (2–5). These studies suggest that cellular responses to receptor tyrosine kinase activation might be dependent upon receptor-selective PTPase activity. A logical extension of this work would be to identify physiological PTPases capable of regulating receptor tyrosine kinase activity. Our recent work involving antisense suppression of the PTPase LAR within intact hepatoma cells has implicated this particular transmembrane PTPase as an important modulator of insulin receptor signaling (6).

The insulin receptor is a heterotetrameric protein complex. Upon binding insulin, the intrinsic tyrosine kinase activity of the transmembrane β subunit increases, allowing it to phosphorylate itself as well as intracellular substrates (reviewed in Ref. 7). Two well characterized substrates of the insulin receptor include insulin receptor substrate 1 (IRS-1) and Shc (8–12). When phosphorylated, IRS-1 can bind to a number of effector proteins, including phosphatidylinositol 3-kinase (PI 3-kinase), the protein tyrosine phosphatase Syp, and the adaptor proteins Grb2 and Nck (reviewed in Refs. 13 and 14). Shc has recently been shown to be the major linkage between the insulin receptor and ras signaling pathways (15–21). Since LAR is expressed by insulin-responsive tissues (fat, muscle, and liver) (1) and has been shown to dephosphorylate selectively tyrosine residues on the insulin receptor critical for tyrosine kinase activity (22), it was a logical PTPase to investigate. When LAR protein levels were suppressed by 63% in the rat hepatoma cell line McA-RH7777, insulin-dependent tyrosine phosphorylation and PI 3-kinase activation were increased by 150% and 350%, respectively (6). The importance of LAR as a modulator of insulin receptor signaling is further supported by recent evidence indicating that increased LAR levels might correlate with insulin resistance in obese human subjects (23).

Insulin-responsive tissues such as liver express receptor tyrosine kinases other than the insulin receptor, such as the epidermal growth factor (EGF) receptor and the hepatocyte growth factor (HGF) receptor. These receptors share many characteristics with the insulin receptor. Upon binding the appropriate ligand, the kinase activity of these receptors increases, resulting in autophosphorylation and intracellular substrate phosphorylation (reviewed in Refs. 24 and 25). Since signaling via these receptors is most likely influenced by the activity of counter-regulatory PTPases, the effect of reduced LAR levels on EGF receptor and HGF receptor signaling was also examined.

EXPERIMENTAL PROCEDURES

Materials—McA-RH7777 rat hepatoma cells were purchased from the American Type Culture Collection (Rockville, MD). The pME4/3 bacteriophage expression vector was a generous gift from Dr. Mark L. Tykocinski (Case Western Reserve University). Phosphotyrosine...
lysate was mixed with a reaction mixture containing 100 mM Tris-HCl, pH 7.4). For the MAP kinase assay, an equal volume of total cell lysate was added to the reaction mixture and incubated for 30 min. The reaction was stopped by adding SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE and analyzed by autoradiography.

Insulin Receptor Tyrosine Kinase Activity—Cell lysates were prepared as described above. Insulin receptors were then partially purified by wheat germ agglutinin affinity chromatography as described (6). PI 3-kinase activity was measured as described (5). Western blot analysis was performed as described previously (5). Western blot analysis was performed as described previously (5).

Growth Factor Stimulation of Cells and Immunoprecipitations—Equivalent numbers of null vector and LAR antisense expressing cells were seeded in 10-cm culture dishes. Following a 24-h incubation in serum-containing Dulbecco’s modified Eagle’s medium, the subconfluent monolayers were incubated for 15 h in serum-free Dulbecco’s modified Eagle’s medium supplemented with 1% bovine serum albumin. Following growth factor treatment, cells were washed twice with phosphate-buffered saline at pH 7.4, harvested in 1.0 ml of lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml benzamidine, 1 mM orthovanadate, 50 mM NaF, 10 mM ATP, and 10 mM tetrasodium pyrophosphate) and homogenized in a hand-driven glass homogenizer. After centrifugation at 10,000 g for 10 min at 4 °C, the clarified supernatants were normalized to protein content (Bio-Rad). Immunoprecipitations were performed as described previously (5). Western blot analysis was performed as described previously (5), except where enhanced chemiluminescence (ECL™) was used. Data generated from Western blot analysis were densitometrically scanned on an XRS image scanner (XRS, Torrance, CA) dedicated to a SUNOS computer (Sun Microsystems, Mountain View, CA).

Insulin receptor tyrosine kinase assay. As shown in Fig. 1, LAR antisense expression resulted in a 3.1-fold increase in insulin receptor tyrosine kinase activity. This increase in PI 3-kinase activation could have resulted if LAR was exerting its effect either at the level of the insulin receptor or a post-receptor substrate, such as IRS-1. To investigate the site of LAR action further, the tyrosine kinase activity of the insulin receptor was examined by using recombinant IRS-1 as the in vitro substrate. Null vector and LAR antisense expressing cells were treated with the indicated concentrations of insulin for 5 min. Receptors were isolated by wheat germ agglutinin affinity chromatography in the presence of phosphatase inhibitors and subjected to a tyrosine kinase assay. As shown in Fig. 1, LAR antisense suppression resulted in a 3.1 ± 0.2 (mean ± S.D.) fold increase in insulin receptor tyrosine kinase activation at 100 nM insulin when compared with null vector expressing cells. Analysis of kinase activity at 1 nM insulin required longer exposures but demonstrated a 2.6 ± 1.1 (mean ± S.D.) fold increase in insulin receptor tyrosine kinase activity in LAR antisense expressing cells. Our previous work demonstrated that a specific 63% reduction in LAR protein levels in the rat hepatoma cell line McA-RH-7777 resulted in a 350% increase in insulin-dependent PI 3-kinase activation (6). This increase in PI 3-kinase activation could have resulted if LAR was exerting its effect either at the level of the insulin receptor or a post-receptor substrate, such as IRS-1. To investigate the site of LAR action further, the tyrosine kinase activity of the insulin receptor was examined by using recombinant IRS-1 as the in vitro substrate. Null vector and LAR antisense expressing cells were treated with the indicated concentrations of insulin for 5 min. Receptors were isolated by wheat germ agglutinin affinity chromatography in the presence of phosphatase inhibitors and subjected to a tyrosine kinase assay. As shown in Fig. 1, LAR antisense suppression resulted in a 3.1 ± 0.2 (mean ± S.D.) fold increase in insulin receptor tyrosine kinase activation at 100 nM insulin when compared with null vector expressing cells. Analysis of kinase activity at 1 nM insulin required longer exposures but demonstrated a 2.6 ± 1.1 (mean ± S.D.) fold increase in insulin receptor tyrosine kinase activity in LAR antisense expressing cells.
ways were examined: IRS-1 and Shc tyrosine phosphorylation. If LAR functions at the level of the insulin receptor, then both insulin-dependent substrate phosphorylations should be increased when LAR levels are suppressed. To investigate the effect of LAR on IRS-1, null vector and LAR antisense expressing cells were treated for 5 min with the indicated concentrations of insulin. After solubilization, the lysates were immunoprecipitated with an IRS-1 antibody. The immunoprecipitated proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride paper. Protein bands were located by enhanced chemiluminescence after incubation with either a phosphotyrosine antibody (4G10) or a p85 PI 3-kinase antibody. A, Western blot analysis of IRS-1 tyrosine phosphorylation. B, Western blot analysis of PI 3-kinase associated with IRS-1. Shown are representative experiments that were each performed at least 3 times.

Through a cascade involving Shc tyrosine phosphorylation and subsequent activation of p31<sup>21<sup>20</sup> (15–21), MAP kinase activation was also examined. Null vector and LAR antisense expressing cells were treated with the indicated concentrations of insulin for 5 min. To directly assess MAP kinase activity, lysates were immunoprecipitated with a MAP kinase antibody. Myelin basic protein was then used as a substrate to measure MAP kinase activity.

MAP kinase is activated by phosphorylation on both tyrosine and threonine residues by MAP kinase (or MEK), which is highly specific for MAP kinase. Since recent work has suggested that mitogen-induced threonine/tyrosine-protein phosphatases can affect MAP kinase activity (31–33), MAP kinase kinase was examined specifically to rule out the possibility that LAR directly affects MAP kinase. As shown in Fig. 3C, suppression of LAR resulted in up to a 4-fold increase in MAP kinase activity. These results demonstrate that reduced LAR levels affected insulin-dependent MAP kinase activation and PI 3-kinase activation to a similar degree, which is consistent with a receptor level site of action.

In addition to its reported in vitro activity toward the insulin receptor, LAR has also been shown to have in vitro activity toward the EGF receptor (34). Since the EGF and HGF receptors are important receptors on hepatocytes, these receptors were examined specifically to determine whether LAR modulates the activity of other receptor tyrosine kinases within the intact cell. McA-RH7777 cells were treated for 1 min with 100 nM insulin and examined by Western blot analysis of PI 3-kinase associated with IRS-1. Shown are representative experiments that were each performed at least 3 times.
ng/ml EGF, 10 ng/ml HGF, or 100 nM insulin. The respective receptors were then immunoprecipitated and examined specifically for phosphoryrosine content by Western blot analysis. As expected, growth factor treatment resulted in an increase in tyrosine phosphorylation for each of the receptors (Fig. 4). When LAR levels were suppressed, however, EGF receptor, HGF receptor, and insulin receptor tyrosine phosphorylation were increased by 3.1 ± 0.4-fold (mean ± S.D.), 2.6 ± 0.2-fold (mean ± 1/2 range), and 1.8 ± 0.2 fold (mean ± S.D.), respectively.

Compatible with a growth factor-dependent increase in receptor tyrosine kinase activation, an HGF-dependent phosphotyrosine substrate of 110 kDa was observed in HGF receptor immunoprecipitates from LAR antisense expressing cells. This receptor-associated band is consistent with previous reports (35). Similarly, a ligand-dependent 55-kDa phosphotyrosine protein (pp55) was observed in EGF receptor immunoprecipitates from LAR antisense expressing cells. A tyrosine-phosphorylated protein of similar molecular weight has been shown to associate with the EGF receptor frequently after EGF treatment (36–38) and has been identified recently as Shc (36, 39).

These HGF-dependent and EGF-dependent substrates were undetectable in immunoprecipitates from null vector expressing cells unless longer exposures were obtained. This is consistent with a stronger ligand-mediated response with suppression of LAR levels. As expected, there were no substrates found within the insulin receptor immunoprecipitates. The predominant substrate of the insulin receptor, insulin receptor substrate 1 (IRS-1), does not associate to an appreciable degree with the insulin receptor. The phosphotyrosine band appearing at 85 kDa has been shown to be a proteolytic fragment of the insulin receptor.

An important post-receptor response to EGF, HGF, and insulin treatment is MAP kinase activation (40–42). As noted above, Shc has been shown to function as a critical factor in MAP kinase activation (12, 17, 18, 21, 43). Since suppressed LAR levels apparently resulted in dramatic increases in EGF receptor-associated Shc/pp55, ligand-dependent MAP kinase activation was anticipated to be increased in the LAR-suppressed cells. When whole cell lysates from LAR antisense expressing cells treated with EGF or HGF were examined by Western blot analysis, growth factor treatment resulted in a decrease in MAP kinase mobility (Fig. 5A). This reduced mobility has been shown to correlate with an increase in phosphorylation (44). The smaller effect of insulin on MAP kinase activation is consistent with previously reported studies that examined the effect of insulin on this pathway in liver parenchyma (36).

Since changes in MAP kinase mobility may not necessarily correlate with changes in activity, MAP kinase activation was measured directly. As shown in Fig. 5B, EGF, HGF, and insulin treatment resulted in MAP kinase activation. Suppression of LAR levels increased this activity by approximately 4-fold for each growth factor tested. To show that the increases in MAP kinase activation were mediated by a pathway that involves MAP kinase kinase, MAP kinase kinase activity was examined. As expected, EGF, HGF, and insulin treatment resulted in increased MAP kinase kinase activation. When LAR levels were suppressed, this activation was also increased by approximately 4-fold for each growth factor tested (Fig. 5C).

If LAR functions by modulating the tyrosine kinase activity of a receptor tyrosine kinase, then all kinase-dependent post-receptor signaling pathways should be increased by reduced LAR levels. To support this premise, another signaling pathway common to EGF, HGF, and insulin receptor signaling, ligand-dependent phosphatidylinositol 3-kinase (PI 3-kinase) activation, was examined. Increases of 257 ± 47% (mean ±
was used as an
levels on the insulin receptor itself. When recombinant IRS-1
than the insulin receptor.
whether LAR can modulate receptor tyrosine kinases other
primarily at the level of the insulin receptor and 2) investigate
the current study were to 1) test the hypothesis that LAR functions
as an important physiological regulator of insulin receptor signaling. The objectives of the current study were to 1) test the hypothesis that LAR functions
by altering the tyrosine kinase activity of
the insulin receptor. This observation is consistent with in vitro
evidence indicating that LAR preferentially dephosphorylates
insulin receptor sites critical for tyrosine kinase activity (22). If
the insulin receptor is the primary target of LAR action, it
would be expected that the LAR-dependent increase in tyrosine
kinase activity would be paralleled by similar increases in
substrate tyrosine phosphorylation. In fact, insulin-dependent
IRS-1 and Shc tyrosine phosphorylation were increased by
approximately 3-fold in LAR antisense expressing cells. As
expected, these increases in IRS-1 and Shc tyrosine phospho-
rylation were propagated further downstream as evidenced by
an increased PI 3-kinase activation and MAP kinase activation.
These data support the conclusion that the insulin receptor itself is a major target of LAR action. The 35% increase in
insulin receptor tyrosine kinase activity previously reported in
LAR antisense expressing cells (6) most likely represents an
underestimate of the actual increase that can be detected with
physiological substrates. Although it is possible that LAR
might play a minor role in directly dephosphorylating either
Shc or IRS-1, the 3-fold increase in insulin receptor tyrosine
kinase activity, as measured with recombinant IRS-1, suggests
that, within the insulin receptor signaling pathway, the insulin
receptor itself is the major site of LAR action. The possibility
that LAR preferentially dephosphorylates certain tyrosine res-
ides on the insulin receptor within the intact cell is being
examined currently.

EGF and HGF are important hepatocyte mitogens that have
been implicated in liver regeneration (reviewed in Ref. 45). HGF has also recently been shown to be essential for liver
development (46). Since the receptors for each of these growth
factors are receptor tyrosine kinases, the effect of LAR on HGF
and EGF receptor signaling was examined. Ligand-dependent
receptor autophosphorylation, substrate phosphorylation,
MAP kinase activation, and PI 3-kinase activation were in-
creased by each of these growth factors. Suppression of LAR
PTPase levels resulted in an amplification of each of these
parameters. This amplification, however, was ligand-dependent;
reduced LAR levels did not affect significantly the basal
activity of any of these receptors. This is best represented by
the PI 3-kinase activation data shown in Fig. 6, where basal PI
3-kinase activation from null vector and LAR antisense ex-
pressing cells was measured at least 12 times. Either directly or
indirectly, LAR appears to function as a factor capable of in-
fluencing cellular signaling via multiple growth factors. Since
signaling from three different receptor tyrosine kinases was
modulated by LAR, this PTPase might function as a general
regulator of ligand-induced tyrosine kinase receptor signaling.
While our conclusions are based on results from one cell line,
McA-RH7777, a recent report by Ahmad et al. (23) suggests
that LAR levels may correlate with insulin resistance in obese
human subjects.

These results raise interesting possibilities as to the mecha-
nism of PTPase action. Since in vitro PTPase specific activity
is 2 to 3 orders of magnitude greater than that of tyrosine
kinase activity (47–49), the accessibility and/or activity of
PTPases must be tightly regulated in order to allow receptor
tyrosine kinase activation. Accessibility might be a function
of receptor tyrosine kinases: after activation, receptors might un-
dergo conformational changes that allow PTPase recruitment.
Alternatively, receptor tyrosine kinases and PTPases might
cluster in specific compartments after receptor activation. Re-
cent evidence has suggested that internal membrane fractions
might represent the critical location for insulin receptor mod-
ation (50). Whether receptor tyrosine kinases and PTPases
are constitutively associated, or associate only after membrane
diffusion, remains to be determined. Although LAR is shown

S.D.) and 314 ± 78% (mean ± S.D.) in EGF-dependent and
insulin-dependent PI 3-kinase activation were observed when
LAR levels were suppressed, respectively (Fig. 6). Although
HGF treatment produced only small increases in PI 3-kinase
activation, suppressed LAR levels consistently yielded an in-
creased signal when compared to null vector expressing cells.
Since PI 3-kinase has been shown to bind to the HGF receptor
(40, 60, 61), it was surprising to observe that HGF treatment
resulted in only a small PI 3-kinase activation under condi-
tions that resulted in a large MAP kinase activation. This interest-
ing finding might be explained by the nature of the HGF receptor's
multifunctional docking site, which is an optimal site for
Grb2 binding and a suboptimal site for PI 3-kinase binding
(40, 61, 62).

**DISCUSSION**

Although the phosphorylations that follow receptor tyro-
side kinase activation have been studied extensively, the mecha-
nism by which the equally important dephosphorylation reac-
tions occur still remains unknown. An important approach to
the question of how receptor tyrosine kinase activity might be
modulated involves the identification of specific physiological
PTPases capable of performing this function. In addition, it is
also important to determine if each receptor tyrosine kinase
is modulated by its own receptor-specific PTPase; alternatively,
a single PTPase could modulate multiple receptor tyrosine ki-
nases. Previous work has demonstrated that antisense inhibi-
tion of the transmembrane PTPase LAR resulted in a 350%
increase in insulin-dependent PI 3-kinase activation (6). This
work was performed in the rat hepatoma cell line McA-
RH7777. Since LAR is normally expressed by hepatocytes,
these studies suggested that LAR is an important physiological
regulator of insulin receptor signaling. The objectives of the
current study were to 1) test the hypothesis that LAR functions
primarily at the level of the insulin receptor and 2) investigate
whether LAR can modulate receptor tyrosine kinases other
than the insulin receptor.

The first parameter examined was the effect of reduced LAR
levels on the insulin receptor itself. When recombinant IRS-1
was used as an in vitro substrate, suppressed LAR levels re-
sulted in a 3-fold increase in insulin-dependent insulin receptor
tyrosine kinase activity. This result supports the hypothesis
that LAR functions by altering the tyrosine kinase activity of

![Figure 6](image-url)
PTPases are important regulators of receptor tyrosine kinases, physiological modulation of these receptors within intact tissues could be dependent on the spatial distribution of LAR. The specificity of LAR for a particular receptor tyrosine kinase might therefore be a function of compartmentalization.

In addition to possible spatiotemporal characteristics of kinase-PTPase interaction, the activity of receptor-selective PTPases might also be regulated tightly. PTPases might become activated only after receptor kinase activation. EGF treatment has, in fact, been shown to increase PTPase activity (51). If LAR’s activity was increased by tyrosine phosphorylation, then the receptor tyrosine kinases examined in this study could be responsible for attenuating their own signals.

The extracellular milieu might also serve an important role in regulating PTPase activity. Recent work demonstrating correlations between increased cell density with increased PTPase activity supports this possibility (53, 54). This increase in PTPase activity might result from the subclass of PTPases that possess extracellular fibronectin type III-like and immunoglobulin-like domains. Two examples of this subclass, R-PTP-κ and R-PTP-μ, have recently been shown to mediate cellular homophilic binding (55–58). Cell–cell contact might cluster PTPases in critical areas of the cell. As the prototype for this type of PTPase (59), LAR might function in a similar manner. Interestingly, LAR is expressed as a complex of two noncovalently linked subunits (63). The 150-kDa extracellular subunit contains fibronectin type III-like and immunoglobulin-like domains. The 85-kDa transmembrane subunit contains a short ectodomain, a transmembrane sequence, and the tandem PTPase catalytic domains (63). LAR has been shown to be shed from HeLa cells during cell growth and after treatment with the protein kinase C activator, phorbol 12-myristate 13-acetate (63). Serra-Pages et al. (64) have shown that this shedding actually involves a secondary proteolytic cleavage in the ectodomain of the 85-kDa subunit. They suggest that shedding of extracellular LAR might alter the biological function of the catalytically active portion by allowing its internalization or relocation within the plasma membrane. Thus, shedding might be one mechanism by which an extracellular stimulus could alter compartmentalization of a PTPase such as LAR. It should be noted that our antisense expressing cells have decreased levels of the 85-kDa LAR subunit. Although we would expect the 150-kDa subunit to be affected similarly since both subunits are derived from the same protein, we cannot formally exclude the possibility that our antisense system differentially affects the 150-kDa and the 85-kDa subunits. This is an area of ongoing investigation.

The results presented in this paper demonstrate for the first time the physiological modulation of multiple tyrosine kinase receptors by a single transmembrane protein-phosphatase. EGF receptor, HGF receptor, and insulin receptor activation were all increased when LAR levels were reduced. Since basal activity was unaltered when LAR levels were suppressed, LAR could function as a specific modulator of ligand-dependent tyrosine kinase receptor activation. In addition, this work supports the general hypothesis that transmembrane PTPases are important regulators of receptor tyrosine kinase signaling.

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