Knock-down of LAR protein tyrosine phosphatase induces insulin resistance

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Abstract To test the role of the leukocyte common antigen-related protein tyrosine phosphatase (LAR) as a regulator of insulin receptor (IR) signalling, an siRNA probe against LAR was developed. Knock-down of LAR induced post-receptor insulin resistance with the insulin-induced activation of PKB/Akt and MAP kinases markedly inhibited. The phosphorylation and dephosphorylation of the IR and insulin receptor substrate (IRS) proteins were unaffected by LAR knock-down. These results identify LAR as a crucial regulator of two key insulin signalling pathways to insulin. Moreover, the siRNA probe provides a molecular tool of general applicability for further dissecting the precise targets and roles of LAR. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Insulin receptor; Protein tyrosine phosphatase; LAR; Protein kinase B; MAP kinase; Insulin resistance

1. Introduction

The leukocyte common antigen-related (LAR) protein is a widely expressed receptor-like protein tyrosine phosphatase (PTP) that has been implicated in the regulation of a diverse range of cell signalling pathways [1].

To facilitate the further study of LAR, we now report the design of an siRNA probe that selectively suppresses LAR expression in the human epithelial-like cell line HEK293. The probe was used to test the role of LAR as a regulator of insulin signalling. The results identified LAR as a key regulator of post-receptor insulin signalling with LAR knock-down inducing insulin resistance.

2. Materials and methods

2.1. siRNA design

The p-Silencer™ 1.0-U6 siRNA expression vector (Ambion, Huntingdon, UK) was used. Potential target sequences for LAR were selected from the mouse database (gi11761807) that were also common to human (gi18860871) and rat (gi9507012). Parameters for selecting siRNA sequences were selected to have a GC content between 45% and 55%, with a sense structure of AA (N19), and TT/TTT/AAAA avoided (terminates transcription). BLAST searches confirmed that the sequences were LAR specific, particularly avoiding of any sequence similarity with closely related LAR-family members PTPε and PTPδ. Four suitable test sequences were selected, corresponding to nucleotide positions 4030, 4224, 5098 and 5170 (named L1, L2, L3 and L4, respectively). The most active of these, L3, was based on the sequence 5'-GGCCTACATACTACACAG-3' and 5'-GGCTACTAACG-3', respectively.

2.2. Cell culture and transfections

Human embryonic kidney (HEK293) cells were obtained from the European Collection of Cell Cultures (LGC Promochem, Teddington, UK). HEK293 Cells were cultured in Dulbecco's modified Eagle's medium: HAM's nutrient medium (50:50), 10% heat inactivated horse serum and 1% t-glutamine (Sigma, Poole, UK), at 37 °C under 5% CO₂. Tissue culture flasks and 12-well plates were pre-coated with 1:10 dilution of poly-L-lysine (Sigma). Cells were transfected using Gene Juice™ transfection reagent (7 μl/2 μg DNA) according to the manufacturer's protocol (Novagen, Nottingham, UK). Cells were transfected at 60–80% confluency.

2.3. Immunoblotting

Immunoblotting was carried out as described previously [2,3]. Where necessary, blots were quantified by densitometric scanning. The anti-PTPα antibody has been described [2]. Other antibodies were from the sources indicated in parentheses: anti-phospho Akt and anti-phospho-p42/44MAPK (NEB, Hitchin, UK); anti-MAP kinase (ERK1, ERK2) (Cambridge Bioscience, Cambridge, UK); anti-insulin receptor (IR) β-subunit and anti-insulin receptor substrate (IRS) antibodies (Upstate, Milton Keynes, UK); anti-LAR and anti-PTP1B (BD Biosciences, Oxford, UK); anti-TC-PTP (Sigma).

3. Results

3.1. Inhibition of LAR expression by siRNA

LAR expression was specifically inhibited using RNAi techniques. The p-Silencer™ vector was used to create siRNA targeted at LAR (see Section 2). Four different constructs (L1–L4) were designed and tested for effectiveness in suppressing LAR expression in HEK293 cells. Twenty-four hours after transfection there was little effect (Fig. 1A), but after 48 h two of the four constructs, L3 and L4, decreased the amount of detectable LAR by 70 ± 2.6% and 59 ± 3.5%, respectively, compared to controls containing transfection reagent only (Fig. 1A and B). The most active construct, L3, was used in all subsequent experiments, and mismatch (MM) and scrambled (SCR) sequences were designed as control siRNAs to test for non-specific effects of the L3 siRNA construct. Neither of the siRNA controls caused decreased LAR expression (Fig. 1B). Immunoblotting
was performed against related PTPs; PTPα, PTPε, PTP1B and TC-PTP. The expression of all four of these PTPs remained unaffected by siRNA treatment (Fig. 1C). The efficacy of the L3 siRNA probe was validated by showing that LAR knockdown markedly increased the tyrosine phosphorylation of endogenous β-catenin [4] by ~5-fold with the mismatch control ineffective (Fig. 1D). Thus, an effective molecular tool for studying LAR has been developed.

3.2. LAR depletion does not alter insulin-induced tyrosine phosphorylation of IR or IRS

Experiments were conducted to rigorously test whether the IR and the IRS-proteins are substrates for LAR in vivo.

The effects of LAR depletion on insulin-stimulated tyrosine phosphorylation of the IR and IRS proteins was examined first. For this, cells were incubated in the presence or absence of 100 nM insulin for 10 min. The IR was detected on anti-phosphotyrosine immunoblots as an insulin-sensitive band at 97 kDa (Fig. 2A(i)). The identity of the IR β subunit was confirmed by the absence of the band after immunoprecipitation with an IR β subunit specific antibody, in comparison to control immunoprecipitations in which the antibody was omitted (results not shown). The antibody used has no reactivity against the insulin-like growth factor (IGF)-I receptor whose β-subunit comigrates with the IR β-subunit. Thus, insulin was acting through the IR and not the IGF-I receptor in our experiments. A strong insulin-sensitive band was also observed at 185 kDa, which after immunoprecipitation were confirmed to be IRS-1 and IRS-2. LAR suppression did not significantly alter the insulin-stimulated tyrosine phosphorylation of either the IR or IRS proteins (Fig. 2A(ii)). To study the effect of LAR depletion on the rate of dephosphorylation of the IR and IRS proteins, time courses of dephosphorylation were determined. Dephosphorylation rates were similar in all the test conditions (Fig. 2B). The effect of LAR knock-down on the steady-state tyrosine phosphorylation of the IR β-subunit and the IRS-proteins over a range of insulin concentrations was also examined. This was important as measurement of the steady-state may be expected to magnify the effects of depleting a PTP that dephosphorylates these substrates. If LAR was controlling the phosphotyrosine status of the IR or IRS proteins, LAR removal would be expected to increase their sensitivity to insulin (i.e., shift the dose–response curve to the left). However, L3 did not significantly affect the level of IR or IRS tyrosine phosphorylation at any of the insulin concentrations tested, when compared to controls (Fig. 2C). These observations suggest that LAR is unlikely to be an important modulator of the tyrosine phosphorylation of IR or IRS in HEK293 cells.

3.3. Effects of density-dependent changes in LAR expression on IR phosphorylation

LAR expression is known to increase with increases in cell density [5,6]. This natural increase in LAR expression provided a further way of testing whether LAR regulates IR tyrosine phosphorylation in vivo. HEK293 cells were seeded at low, medium and high cell densities and harvested 18 h later, having reached confluencies of 25%, 45–50% and 95–100%, respectively. LAR expression was 3.5-fold higher in the most confluent cells when compared to low density cells (Fig. 3A). IR expression also increased and was 2-fold higher in the more

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Fig. 1. Specific inhibition of LAR expression in HEK293 cells by siRNA. (A) Anti-LAR blot. Cells were analysed 24 and 48 h after transfection with four different siRNA constructs, targeted against LAR (L1–L4), or treatment with transfection reagent alone (GJC). (B) Anti-LAR blot showing LAR expression in cells treated for 48 h with transfection reagent alone (GJC), L3, L4, L3-mismatch (MM) or L3-scrambled (SCR) siRNA constructs. Blot shown is representative of at least four independent experiments. (C) As B except cells were immunoblotted for other major PTPs, including, PTPα, PTPε, PTP1B and TC-PTP. (D) Phosphotyrosine (pY) immunoblot of immunopurified β-catenin from cells treated for 48 h with transfection reagent (GJC), L3 siRNA and L3-mismatch (MM) constructs. Total β-catenin and LAR immunoblots from the same samples are shown to indicate equal loading and knock-down of LAR, respectively. Immunoblots shown are representative of two individual experiments.
Fig. 2. LAR depletion does not alter insulin-induced tyrosine phosphorylation of IR or IRS proteins. (A): (i) Anti-phosphotyrosine blot showing tyrosine phosphorylation of the IR and IRS proteins before and after stimulation with 100 nM insulin for 10 min. HEK293 cells were treated with transfection reagent only (GJC), L3, L3-mismatch (MM) or L3-scrambled (SCR) siRNA sequences. Blots confirmed extensive depletion of LAR by L3 (72 ± 5%) with no depletion of LAR in the controls. Results are representative of at least 4 experiments. (ii) Histogram representation of fold-increases in the tyrosine phosphorylation of the IR and IRS proteins from above experiments, as determined by densitometry. Transfection condition is indicated on the graph. (B) Anti-phosphotyrosine blots showing rates of dephosphorylation of the IR and IRS proteins. Following 10 min stimulation with 100 nM insulin, the medium was removed and replaced with serum free medium. Dephosphorylation was allowed to proceed for 0, 1, 3, 5 and 7 min: (i) cells treated with transfection reagent only (GJC); (ii) cells treated with L3. Blots confirmed extensive depletion of LAR by L3 (65 ± 6%). (C) Dose–response curves for tyrosine phosphorylation of the IR β-subunit and IRS-proteins. Cells were treated with transfection reagent only (GJC, □) or L3 (■), and incubated with 0, 1, 10, 50 or 100 nM insulin for 10 min. The extents of tyrosine phosphorylation of the IR (top two lines) and IRS proteins (lower two lines) were determined by densitometry from anti-phosphotyrosine immunobLOTS. LAR knockout by L3 was 74 ± 5%.

Fig. 3. Effects of density-dependent changes in LAR expression on insulin signalling. (A) Anti-LAR blot of cells grown at low (25%), medium (45–50%) and high (95–100%) cells densities. Equal amounts of protein were loaded in each lane. (B) Anti-phosphotyrosine blot of low and high density cells before and after stimulation with 100 nM insulin for 10 min, showing insulin-induced increases in tyrosine phosphorylation of the IR and IRS proteins. (C) Corresponding blots against the IR β-subunit. ImmunobLOTS representative of at least 3 independent experiments.
confluent cells (Fig. 3C). Increases in IR expression with increased cell confluency have not been previously reported. The increased IR expression was matched by an increase in the level of insulin-stimulated tyrosine phosphorylation of the IR of 1.95-fold (Fig. 3B). Thus, natural increases in endogenous LAR expression did not inhibit IR tyrosine phosphorylation. These results independently confirm those obtained by siRNA knock-down.

3.4. LAR knock-down induces insulin resistance

Knock-down of LAR by the L3 siRNA probe markedly inhibited the insulin-stimulated increase in the phosphorylation of protein kinase B (PKB, also called Akt) on serine 473 by >90% (Fig. 4A). Control mismatch and scrambled siRNAs were without effect. Phosphorylation of PKB on serine 473 is well known to reflect the activity of the kinase. Total PKB levels were not altered by any transfection condition (Fig. 4A). Furthermore, the PI3-kinase-independent phosphorylation of MAP kinase in response to insulin was virtually abolished in LAR-knock-down cells (Fig. 4B). Again, control mismatch and scrambled siRNAs were without effect and the total amount of MAP kinase was not altered by any transfection condition (Fig. 4B). These results show that LAR knock-down has a profound inhibitory effect on the signalling by the two major pathways utilised by insulin with removal of LAR inducing insulin resistance. The insulin resistance induced by removal of LAR was genuine insulin resistance because signalling responses to serum growth factors were unaffected, e.g., there was no decrease in DNA synthesis in response to serum growth factors, a response that is known to be transduced by the MAP kinase pathway (data not shown) [3].

4. Discussion

LAR is a transmembrane receptor-like PTP with a wide tissue distribution. It has been implicated in the regulation of various signalling pathways triggered by growth factors, such as insulin [1]. To facilitate the study of the signalling role of LAR, we now report the development of an siRNA probe that targets LAR. The siRNA probe achieved 70% knock-down of LAR in HEK293 cells (Fig. 1). Other PTPs, such as PTPα, PTPη, PTP1B or TC-PTP, that have also been implicated in the regulation of growth factor receptor signalling, were not depleted by the siRNA probe. The siRNA probe was then used to test the role of LAR in regulating insulin signalling.

Knock-down of LAR induced post-receptor insulin resistance with the insulin-induced activation of PKB/Akt and MAP kinases markedly inhibited in the absence of effects on the phosphorylation or dephosphorylation of the IR or IRS proteins. The insulin resistance induced by removal of LAR was genuine insulin resistance because signalling responses to serum growth factors were unaffected (data not shown), e.g., there was no decrease in DNA synthesis in response to serum growth factors, a response that is known to be transduced by the MAP kinase pathway [3]. Our results identify LAR as a regulator of the sensitivity of two key insulin signalling pathways to insulin. Moreover, the siRNA probe provides a molecular tool of general applicability for further dissecting the precise targets and roles of LAR.

Our results parallel the observation that expression of a catalytically inactive PTP, Shp-2, blocked the insulin-stimulated activation of MAP kinase [7]. Shp-2 appeared to act by targeting a protein downstream of p21ras [7].

Whether LAR dephosphorylates the IR has been an area of controversy with initial studies in model cell systems in favour of such a role [8,9]. Thus, antisense suppression of LAR, to a similar level achieved by our siRNA probe, increased IR auto-phosphorylation and reduced the rate of IR dephosphorylation in rat hepatoma cells [8,9]. The difference between these results and those herein may be due to use of different cell types or that the long antisense sequence (546 bases) imposed non-specific knockout effects on PTPs besides LAR. More recently, two different transgenic mice studies failed to find evidence that LAR acts on the IR. In one study insulin-stimulated hepatic tyrosine phosphorylation of the IR and IRS-1 was not significantly increased in LAR (−/−) mice compared to wild type animals [10]. In another mouse model where LAR was overexpressed in muscle there was no change in the tyrosine phosphorylation of either the IR or IRS-1 [11]. For the first time, our results support the transgenic mice studies in a model cell system.

The insulin resistance induced by siRNA triggered LAR knock-down is supported by post-receptor signalling defects observed in a LAR knockout mice study [10]. These mice displayed significant resistance to the effects of insulin at the PI3-kinase level. However, the significance of the LAR knockout mice results alone was uncertain because of the potential for changes in the expression of an array of proteins in the long term knockout models. Consequently, it was unclear whether...
the insulin resistance in the LAR knockout mice was directly attributable to removal of LAR or a secondary or compensatory effect. Our results now clearly show in an isolated cell system that direct removal of LAR in the short term induces insulin resistance and thereby establish a new role for LAR. A major challenge for the future will be to identify the targets for LAR that mediate this effect.

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References


