SARS-COV2 Envelope Protein (E) interacts with the Lysophosphatidic Acid Receptor 1 (LPAR1) from humans

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Abstract

A proteomic screen of human proteins interacting with the SARS-COV2 Envelope (E) protein identified LPAR1 as a strong candidate. Physical association of E protein and LPAR1 was confirmed by co-immunoprecipitation and cell surface staining. LPAR1-E protein interaction was confirmed in all eight human cell lines tested. Many additional proteins participating in the E protein interactions network were also enriched from each of the cell lines, some of which were cell type specific. These findings suggest that LPAR1 is likely a cell surface receptor for the E protein, and pave the way for follow-on studies aimed at understanding the biological significance of the interactions in SARS-COV disease, including the signaling mechanisms.

Introduction

The Envelope (E) protein of SARS-COV2 is a small, transmembrane protein critical for virus life cycle. As a viroporin, it self-assembles in cell membranes forming pentameric protein-lipid pores to allow ion transport across cellular membranes. It has been suggested that interactions between the cytosolically oriented C-terminal end of the E protein and the C-terminal ends of the M or S proteins of SARS-COV2 are important for infection. Besides the channel activity, the E protein is also targeted to Golgi membranes and needed for virion morphogenesis and release. Despite these advances, the precise role of the E protein in SARS-COV2 infection has remained enigmatic.

This report describes the *in vitro* protein interactions network of E protein in human cells. Proteomic analysis of a B cell line identified several cell surface receptor candidates, including one protein exhibiting a strong interaction with the E protein. This protein was identified as LPAR1, a member of the G protein coupled receptor family. LPAR1, and its interaction with the E protein, were confirmed by physical association of the E protein to the cultured human cells, enriched by co-precipitation with E protein, and co-localized with LPAR1 from each of seven additional cell lines of differing cell lineages.

LPAR1 is one of six Lysophospatidic acid receptors (LPAR1 - LPAR6) [1] encoded in the human genome, and a few additional LPAR-like proteins have been proposed. LPAR receptors contain transmembrane domains for ligand binding and signaling in human vascular, reproductive, nervous, and immune systems. LPA levels and LPAR receptor signaling mechanisms have been implicated in human airway disease, which is also a co-morbid condition in SARS-COV2 infection. However, it is not clear if the E protein interacts with cellular membrane components, involved in receptor signaling, or interferes with lipid metabolism to alter membrane structure or function. The potential for evaluating the E protein as a vaccine candidate and implications for SARS-COV disease therapy involving the E protein-LPAR1 signaling mechanisms are discussed.

Results

SARS-COV2 Envelope protein (E protein) interacts with human cellular proteins in a cell-type specific manner. To identify human proteins interacting with the E protein, several human cell lines were analyzed by Western blotting using a biotinylated, bacterial recombinant E protein, and whole cell extracts from cultured cells [Fig. 1a]. A prominent band of around 42 kda was observed in all four human samples tested. Several other bands were common between the cells, although the relative intensities of some varied from one cell type and another as well as with the 42 kda band. A triplet of bands between ~14 to ~ 17 kda exhibited strong interactions with the E protein, and was unique to the DOHH2 (Diffuse large B-cell lymphoma line) sample. A control cell line, COS7, exhibited a pattern of interacting proteins different from any of the human cells. These observations suggest that the E protein interacts with multiple proteins from human cells, and that the interacting proteins vary with the cell type.

Protein interactions network of the E protein in DOHH2 cells. A STRINGseeker assay was performed as described previously [2], using the bacterially encoded E protein as bait and total cellular proteins from DOHH2 cells as preys. As shown in Fig1b, many proteins were enriched by the assay, including the ~42 kda protein shown in Fig. 1a. Barring minor alterations to protein mobilities of STRINGseeker products, there was good congruence with the pattern of bands observed by Western blotting and STRINGseeker purified proteins. Overall, the relative ratios of proteins in the STRINGseeker sample varied much less compared to the same in whole cell extracts, suggesting a degree of normalization of their concentrations. Network participation of the proteins and the required stoichiometry of binary interactions or complex formation within the networks are expected to promote normalization, which in turn will influence their enrichment.

Identification of a candidate cell surface protein associating with the E protein *in vitro*. Preliminary analysis of the E-STRINGseeker products from DOHH2 cells identified several cell surface proteins, suggesting that the E protein may possess a cell surface binding property. Further proteomic analysis of the sample showed that the most prominent among the cell surface proteins was Lysophosphatidic Acid Receptor 1 (LPAR1). The known molecular mass of LPAR1 correlated well with the ~42 kda band observed in Western blots.

LPAR1 is a clinical target in idiopathic pulmonary fibrosis, a chronic, progressive, and fatal fibrosing lung disease [3-7]. A member of the G-protein coupled receptors family, it is one of a family of receptors for Lysophosphatidic acid (LPA), mediating LPA signaling. LPAR1 contributes to the responses to tissue damage and infectious agents, with prominent roles in chemotaxis, cell migration, responses to injury, and pain perception, as well as triggers inflammation in response to bacterial lipopolysaccharide. LPAR1 has been shown to be expressed in many tissues, including abundantly in the brain and lung.

Binding of the E protein to the cell membrane is competed by LPAR1 antibody. A commercially available antibody to LPAR1 was employed for detecting LPAR1 by Western analysis in each of 8 human cell lines tested (Fig. 2a). The most prominent band was of ~42 kda protein, but 2 additional larger isoforms were variably present in different cell lines. In most cells other than DOHH2, LPAR1 was observed as a doublet of closely migrating species, which likely represent modified forms of the protein. Human AGTR1 and

AGTR2 are also ~42 kda in size. The LPAR1 42kda protein co-migrates with AGTR2 (Fig. 2b), but is different from it since the larger bands in each lane exhibit different sizes as compared to those in Fig. 2a. Taken together, these observations confirm the expression of LPAR1 in all the cell lines tested.

The binding of the E protein to membrane associated LPAR1 was tested. The 8 human cell lines were grown to 90% confluence in 96-well plates, fixed with formalin, and probed with a biotinylated E protein, with or without competition with unlabeled LPAR1 antibody. As shown in Fig. 3a., 7 of the 8 cell lines showed robust signals for cell surface binding of the E protein. The E protein binding signal was diminished by 10% to 43% in presence of 80-fold molar excess of LPAR1 antibody in different cell lines. HEK293 and HEK293T cells exhibited nearly 30% reduction in signal intensities, and HeLa cells nearly 45%. Surprisingly, 2 of the 4 lung cell lines, H727 and H460 showed only a modest inhibition by LPAR1 antibody (~11%), while in a third line, H1581, LPAR1 antibody did not compete the binding of the E protein. Taken together, the western and cell staining data demonstrate association of the E protein to cell surfaces, likely mediated by LPAR1.

Under the same conditions, the S protein probe showed negligible competition by LPAR1 antibody, although the signal intensities were significantly higher signal strengths (Fig. 3b). The higher signal strengths with the S protein may be ascribed to its association with AGTR1, AGTR2, or both. Intriguingly, cells which exhibited little or no inhibition of E protein binding by LPAR1 antibody, namely H460, H1581, and HepG2, exhibited modest inhibitions (9% to 14%) with the S protein probe. The reasons for this are not clear. Perhaps, steric effects, collaboration with other membrane-bound receptors, membrane topology, or cell-specific modifications of LPAR1 may account for these differences.

The LPAR1 antibody was titrated over a 1,600-fold range for competition with the surface binding of the E protein to each of the 8 cell lines (Fig. 3d). All the cells, except HepG2, showed a dose response of inhibition of surface binding. E protein binding was reduced more than 60% in these cells at either the 400x or 1600x molar excess of LPAR1. The lack of inhibition with HepG2 cells is likely not due to absence of LPAR1 in these cells, but more so on account of its growth in multiple layers in culture, which may require different conditions for staining.

Co-purification and co-localization of LPAR1 with E interacting proteins: To confirm LPAR1 interaction with the E protein *in vitro*, STRINGseeker assays were performed with each of the 8 cells lines using the recombinant E protein as bait. The interacting protein products were run alongside the corresponding whole cell extracts and probed by Western analysis. LPAR1 was detected using LPAR1 antibody (Fig. 4a) or co-localized with the ~42 kda protein using E protein as probe (Fig. 4b).

As shown in Fig. 4a., LPAR1 was detected in all 4 STRINGseeker samples tested, albeit with different degrees of enrichment. As with DOHH2 cells, H1975 and H460 cells showed substantial enrichments of LPAR1. LPAR1 recovery was lower in HEK293 or H1581 STRINGseeker samples, although the corresponding cell extracts showed abundant LPAR1 protein. The reasons for these are not clear. Perhaps, a competing interacting protein or cell-specific modifications of LPAR1 may affect complexing

of LPAR1 with the E protein in the STRINGseeker assays. STRINGseeker products from the other 4 cell types were not analyzed.

Another set of samples was probed with the E protein (Fig. 4b). As observed previously with DOHH2, multiple interacting proteins were observed in the cell extracts and enriched by STRINGseeker, including, in particular, the 42 kda band observed previously. The same band was detected in STRINGseeker products from DOHH2 cells, HEK293T, and HepG2, albeit with lower intensities in the latter two samples.

To investigate if LPAR1 also interacted with the S protein, STRINGseeker assays were performed with all 8 cells using the S protein as bait, and analyzed as above. As shown in Fig. 4c., LPAR1 antibody failed to detect any band in the STRINGseeker products, nor did a ~42 kda protein co-purify with them. Many proteins ranging in size from 20 kda to over 200 kda were enriched in all STRINGseeker products with the S protein (not shown); however, a protein co-migrating with LPAR1 was not one of them. The observations suggest that the interaction of LPAR1 is specific to the E protein.

Conclusions

This report describes the identification of the Lysophosphatidic acid receptor 1 (LPAR1) as an *in vitro* interacting protein of the SARS-COV2 Envelope (E) protein. While in the initial screen LPAR1 was highly enriched by interaction with the E protein using STRINGseeker, cells of hepatic, cervical, lung, and kidney origins also exhibit the E protein and LPAR1 interaction, suggesting it to be of wide occurrence in many human cell types.

Association of the E protein with cell membrane bound LPAR1 and co-purification with E protein interacting proteins from the eight cell lines tested strongly suggest that LPAR1 is an interactant of the E protein in humans. Heterogeneity of other proteins co-purifying with LPAR1 suggests the existence of multiple, cell-specific, signaling mechanisms and may bear some relevance to the multiple clinical manifestations of SARS-COV infections in humans. DOHH2, H1975, and H460 exhibited the strongest interactions while HEK293 and HEK293T cells showed noticeably lower recoveries.

It remains to be seen if any LPAR receptors other than LPAR1 was also enriched in the assays. However, the strong signature with LPAR1 suggests a key interaction with the E protein and LPAR1. Further studies with mapping the interacting regions of the two proteins can help characterize the interaction, and contributions from any other LPAR receptors. Mass spectrometry analysis of the STRINGseeker products from the 8 cell types can help identify any additional LPA receptors, or collaboration with other receptors [8].

Extension of these studies in SARS-COV infected cells can help understand the clinical significance of E protein interactions and further delineate molecular mechanisms of SARS-COV2 virus pathogenesis [9]. It remains to be determined if virus production is at all needed for the E protein association with LPAR1 in clinical samples, or if the protein, or its fragment, functioning as an exotoxin (such as Diphtheria toxin) is sufficient for clinical symptoms to occur, including lung abnormalities. Determining the detailed

molecular signatures of the STRINGseeker products and assigning some of the proteins to well-studied signaling pathways may provide valuable clues, and a mechanistic response, of the E protein associating with the LPAR1 protein network.

Methods

Cells: DOHH2 were purchased from Creative Biomart). HEK293, HEK293T, HepG2, HeLa, NCI-H1581, NCI-H460, NCI-H727 and NCI-H1975 cell lines were purchased from ATCC. Cells were maintained in culture according to manufacturers' recommended protocols.

Sample preparation: Cells were grown to 80% to 90% confluence, scraped, and washed once with cold PBS. Approximately 4×10^6 cells were resuspended in 400 ul of 1 x Cell Lysis buffer (9803, Cell Signaling Technology) and incubated at room temperature for 15 min with occasional mixing. Lysates were cleared by centrifugation and the supernatants stored in aliquots at -20 °C. The protein concentrations of WCEs were determined at 280 nm with a NanoDrop Spectrophotometer.

Antibodies & Reagents: Recombinant 2019-nCoV Envelope protein (RP01263LQ) was purchased from AbClonal Technology and recombinant SARS-COV2 Spike protein (Z03483) from GenScript. Mouse monoclonal antibody for LPAR1 was purchased from Santa Cruz Biotechnology; GAPDH was purchased from ProteinTech, and rabbit polyclonal antibody for AGTR2 was a kind gift from AbClonal.

Western blotting: 20 ug of total proteins WCE or 20 ul of STRINGseeker products were loaded per lane on a 4-20 % gradient PAGE (GenScript) or 12 % Bis-Tris gels (Invitrogen) under denaturing conditions. The proteins were transferred to Immobilon-FL transfer membranes (Merck Millipore Ltd.) for 2 hours at room temperature. Filters were washed once in TBS, blocked for 2 hr in Intercept Blocking Solution (Li-Cor), and then incubated for 2 hours with biotinylated probe or antibody in Intercept Antibody diluent (Li-Cor) at a dilution of 1:2,000. After washing 3x with 3 changes of PBST at room temperature, the filters were incubated for 1 hr with 1:15,000 fold dilution of IR Dye 800 Streptavidin or IRDye® 680LT Goat anti-Mouse IgG Secondary Antibody (Li-Cor). Filters were washed with PBST and scanned in the Odyssey 9120 at 700 nm (Alexa) or 800 nm (Streptavidin). Images from Odyssey 9120 were analyzed with the Image Studio Lite software as per manufacturers protocols.

Declarations

Competing interests: The author declares no competing interests.

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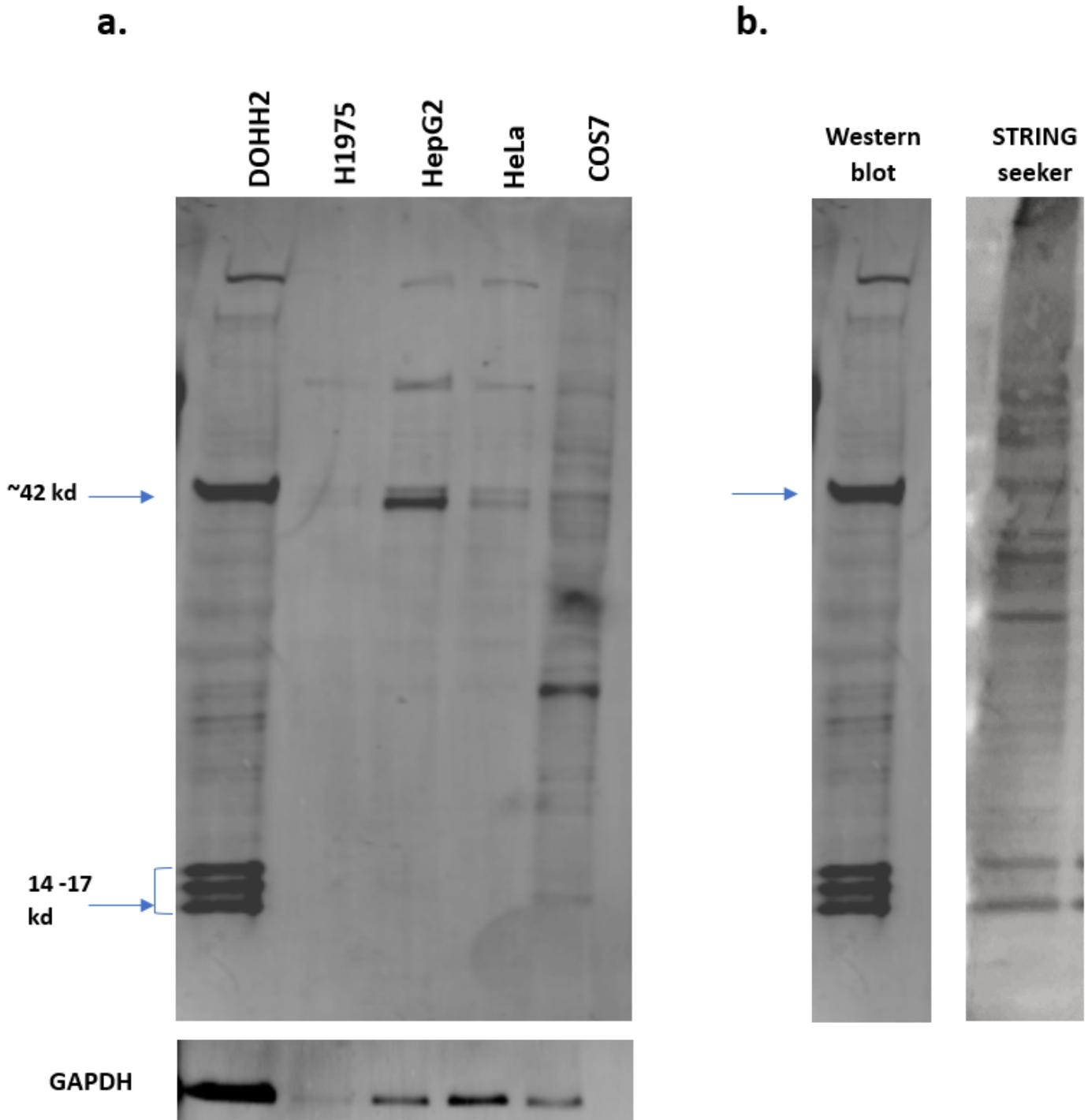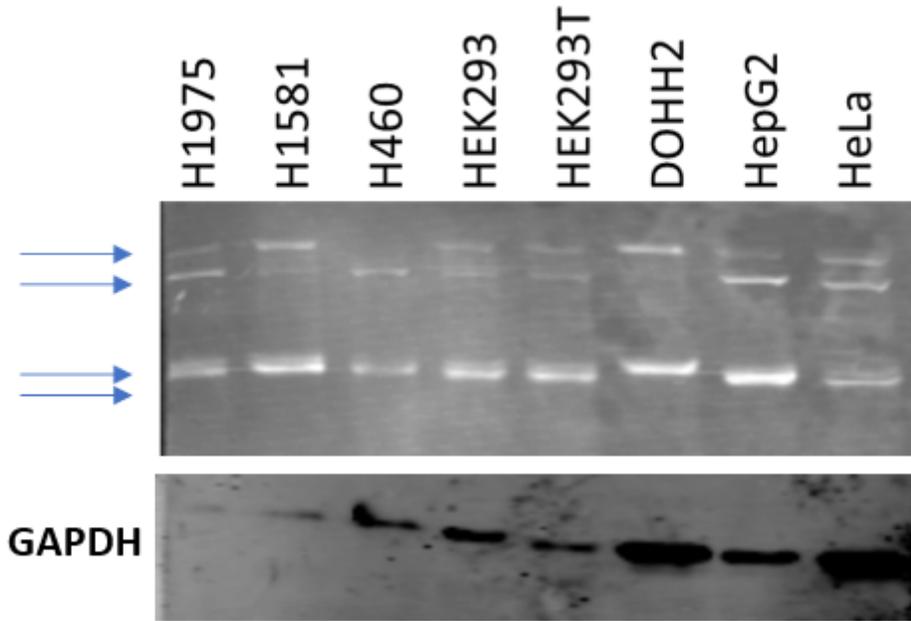
Figures

Figure 1

E protein interacts with human cellular proteins in a cell-type specific manner.

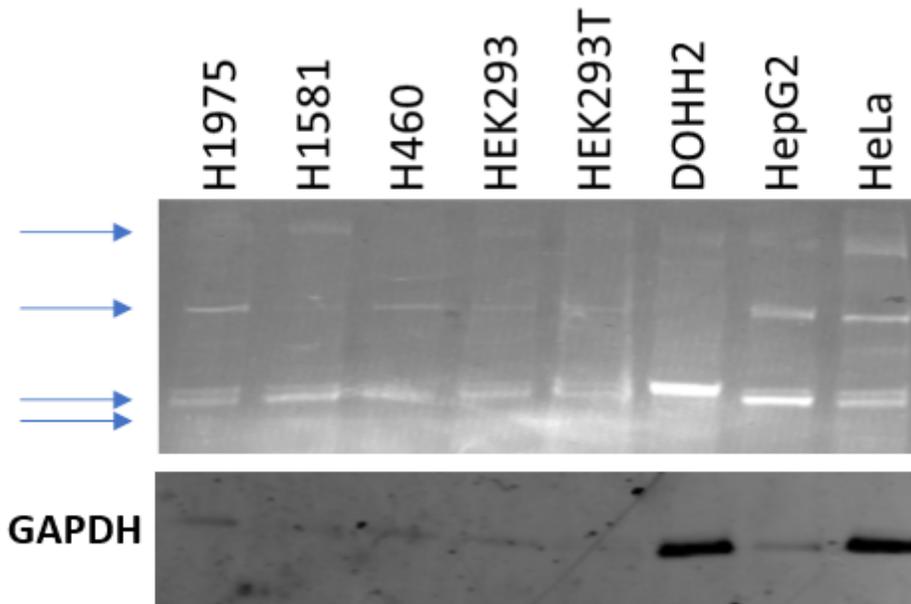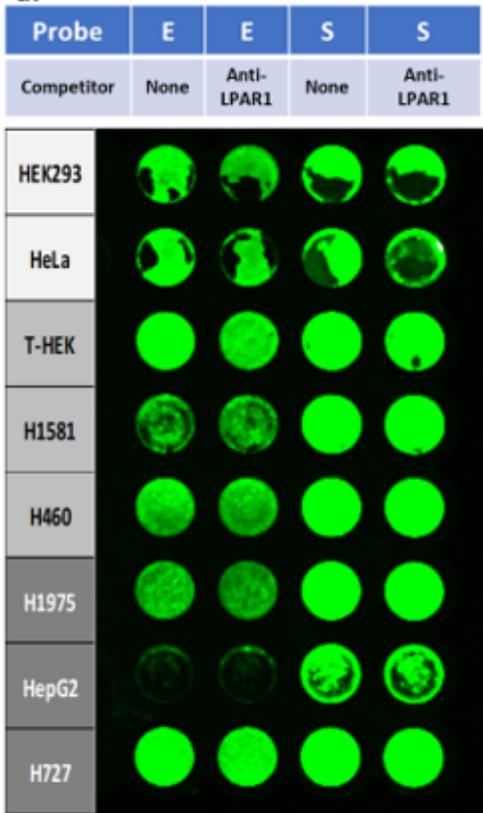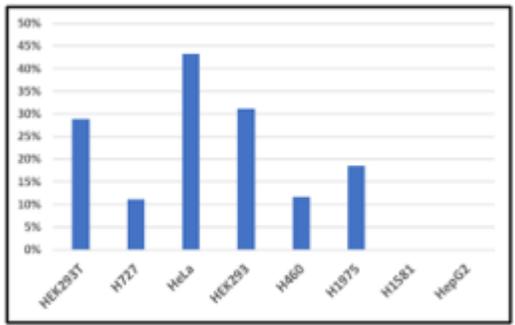**a. Anti-LPAR1 probe**

b. Anti-AGTR2 probe
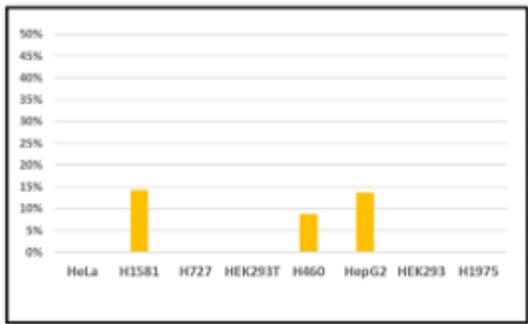
Figure 2

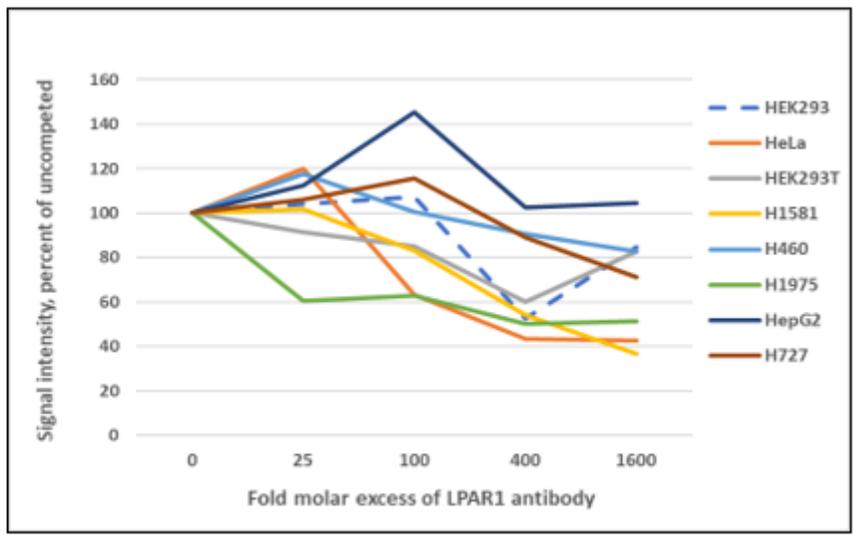Expression of LPAR1 in human cell lines.

Figure 3

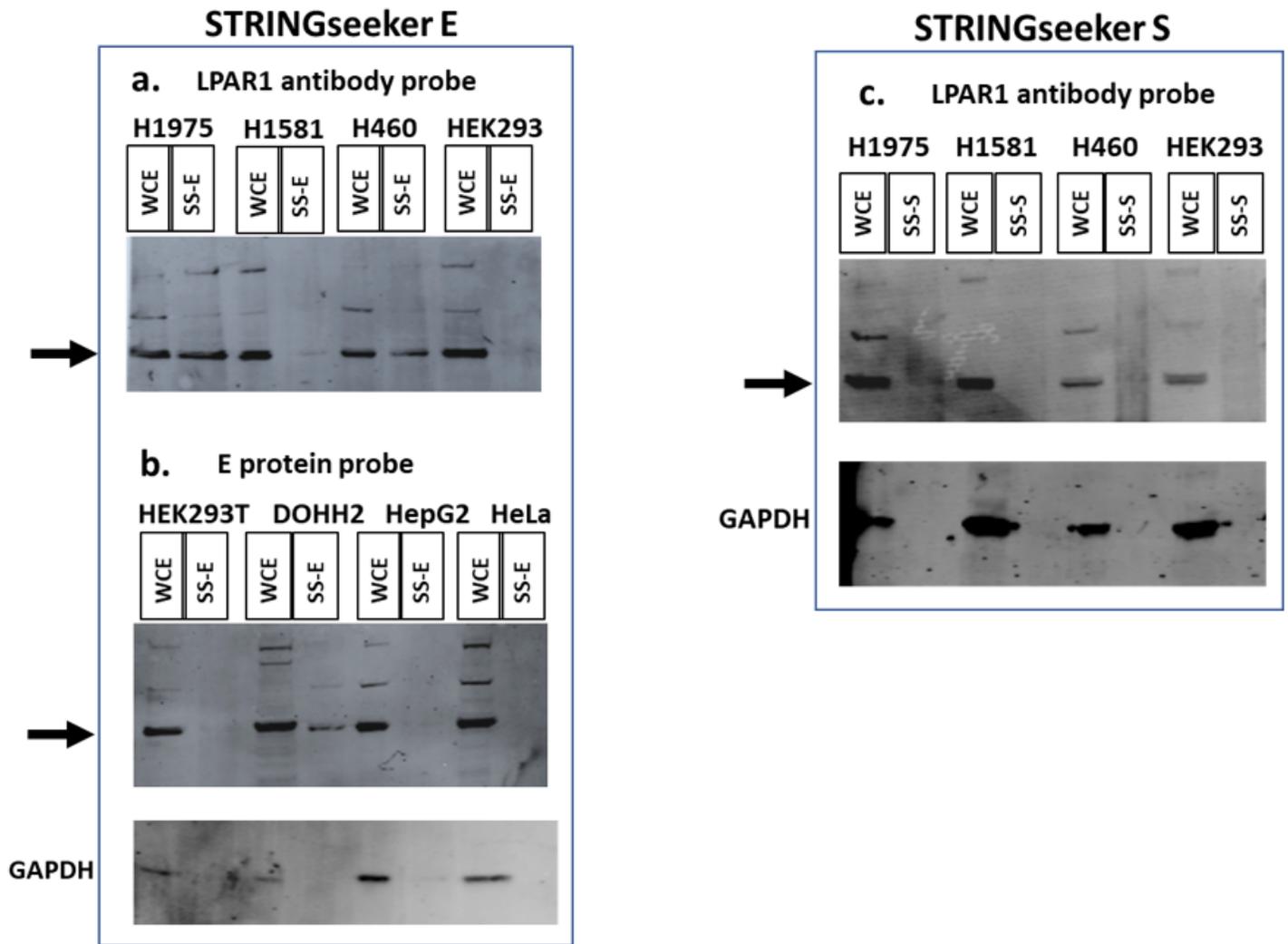LPAR1 antibody inhibits binding of E protein to cells in vitro.

Figure 4

LPAR1 physically associates with the E protein in vitro and co-purifies with STRINGseeker products.