The Functional Role of the Second NPXY Motif of the LRP1 \(\beta\)-Chain in Tissue-type Plasminogen Activator-mediated Activation of N-Methyl-D-aspartate Receptors*\[5]\[5]

Received for publication, September 11, 2007, and in revised form, February 11, 2008. Published, JBC Papers in Press, March 5, 2008, DOI 10.1074/jbc.M707607200

Anne M. Martin\[1], Christoph Kuhlmann\[1], Svenja Trossbach\[1], Sebastian Jaeger\[1], Elaine Waldron\[1], Anton Roebroek\[1], Heiko J. Luhmann\[1], Alexander Laatsch\[2], Sascha Weggen\[2], Volkmar Lessmann\[2], and Claus U. Pietrzik \[1]\[1]

From the \[1]Institute of Physiological Chemistry and Pathobiology, Molecular Neurodegeneration and \[5]Institute of Physiology, Johannes-Gutenberg-University Mainz, D-55099 Mainz, Germany, \[6]Experimental Mouse Genetics, Department of Human Genetics, KU Leuven and Flanders Institute for Biotechnology, B-3000 Leuven, Belgium, the \[1]Department of Biochemistry and Molecular Biology II: Molecular Cell Biology, University Medical Center Hamburg-Eppendorf, D-20246 Hamburg, Germany, \[7]Institute of Neuropathology, Department of Molecular Neuropathology, University Medical Center Heinrich-Heine-University, D-40225 Düsseldorf, Germany and \[11]Institute of Physiology, Otto-von-Guericke-University, D-39120 Magdeburg, Germany

The low density lipoprotein receptor-related protein 1 (LRP1) emerges to play fundamental roles in cellular signaling pathways in the brain. One of its prominent ligands is the serine proteinase tissue-type plasminogen activator (tPA), which has been shown to act as a key activator of neuronal mitogen-activated protein kinase pathways via the \(N\)-methyl-\(d\)-aspartate (NMDA) receptor. However, here we set out to examine whether LRP1 and the NMDA receptor might eventually act in a combined fashion to mediate tPA downstream signaling. By blocking tPA from binding to LRP1 using the receptor-associated protein, we were able to completely inhibit NMDA receptor activation. Additionally, inhibition of NMDA receptor calcium influx with MK-801 resulted in dramatic reduction of tPA-mediated downstream signaling. This indicates a functional interaction between the two receptors, since both experimental approaches resulted in strongly reduced calcium influx and Erk1/2 phosphorylation. Additionally, we were able to inhibit Erk1/2 activation by competing for the LRP1 C-terminal binding motif with a truncated PSD95 construct resembling its PDZ III domain. Furthermore, we identified the distal NPXY amino acid motif in the C terminus of LRP1 as the crucial element for LRP1-NMDA receptor interaction via the adaptor protein PSD95. These results provide new insights into the mechanism of a tPA-induced, LRP1-mediated gating mechanism for NMDA receptors with its highest expression in liver and brain. Following its synthesis in the endoplasmic reticulum as a 600-kDa type I transmembrane glycoprotein, LRP1 is cleaved in the Golgi compartment by furin, producing two subunits, 515 and 85 kDa in size. These two subunits remain noncovalently associated during their transport to the cell surface (1). Via a receptor-recycling pathway, LRP1 is responsible for the endocytosis of more than 30 different extracellular ligands (2, 3). In neurons, where it is highly expressed and predominantly localized in neuronal cell bodies and dendritic processes (4), LRP1 is a major receptor for apoE/lipoprotein-containing particles and tissue-type plasminogen activator (tPA)\[2]. In addition to its role in endocytosis of various ligands, LRP1 has been implicated to play a crucial role in cell signaling. The observation that the LRP1 C terminus undergoes rapid tyrosine phosphorylation after tPA binding corroborates its function as a signal transmitter (6). Several adaptor proteins can bind to the LRP1 tail, some of which seem to play a role in neuronal calcium, platelet-derived growth factor, or MAPK signaling (7–9). Beyond its known function of lipid uptake, LRP1 has also been attributed to be the mediator of an LTP-enhancing effect of exogenously added tPA in hippocampal slices from tPA-deficient mice (5). Further evidence supporting the hypothesis of an involvement in synaptic plasticity results from neuron-specific LRP1 knock-out mice, which show motor and coordination defects, muscle tremor, premature aging, and death (4). Given the central role of \(N\)-methyl-\(d\)-aspartate (NMDA) receptors in synaptic plasticity and the function of LRP1 as a tPA receptor, an interaction between both molecules seems likely. Interestingly, a possible interaction between LRP1 and the NMDA receptor has been deduced from immunohistochemical and co-immunoprecipitation experiments, pointing to an LRP1-NMDA receptor interaction at the synapse (4). In contrast to these findings, it has been reported that tPA binds directly to the NMDA receptor, resulting in the

---

* This work was supported by Deutsche Forschungsgemeinschaft (DFG) Grant Pi379 3-3 (to C. U. P.), Stiftung Rheinland-Pfalz (to C. U. P.), and DFG Sonderforschungsbereich (to V. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

† To whom correspondence should be addressed: Institute of Physiological Chemistry and Pathobiology, Molecular Neurodegeneration, Johannes-Gutenberg-University Mainz, Duesbergweg 6, D-55099 Mainz, Germany. Tel.: 49-6131-3925390; Fax: 49-6131-3926844; E-mail: pietrzik@uni-mainz.de.

‡ The abbreviations used are: tPA, tissue-type plasminogen activator; RAP, receptor-associated protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Erk1/2, extracellular signal-regulated kinase 1 and 2; shRNA, small hairpin RNA; siRNA, small interfering RNA; P-Erk1/2, phospho-Erk1/2; NMDA, \(N\)-methyl-\(d\)-aspartate; stPA, synthetic recombinant tPA; NPXY(2), distal NPXY motif.
cleavage of the NMDA receptor subunit 1, leading to enhanced NMDA receptor-mediated intracellular calcium accumulation and neuronal degeneration (10). Other studies report a proteolysis-independent effect of tPA on NMDA receptor-mediated downstream signaling events through direct interaction of tPA with the NMDA receptor (11). Although the underlying mechanism, how tPA facilitates NMDA receptor activation, remains controversial, the general involvement of tPA in modulating NMDA receptor function is not in doubt (12, 13).

In this study, we show that tPA indeed activates the NMDA receptor cascade as hypothesized before (14). However, we are able to demonstrate that tPA-induced NMDA receptor activation and downstream signaling is exclusively mediated via LRP1. This activation is based on a mechanism involving the distal NPXY motif of LRP1 and the adaptor protein PSD95, which bridges LRP1 to the NMDA receptor. This complex formation is followed by calcium influx into the cell and subsequent activation of the MAPK pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant tPA derived from Chinese hamster ovary cells (Actilyse) was purchased from Boehringer Ingelheim, and pure synthetic recombinant human tPA (stPA) was purchased from Biopur (Bubendorf, Switzerland). The NMDA receptor blockers DL-AP5 (Sigma) and MK-801 (Sigma) were dissolved in 0.1 M NaOH solution and in double-distilled H2O, respectively. The MEK inhibitor U0126 (Calbiochem) was diluted from a stock solution of 10 mM in DMSO.

We constructed a RAP-GST fusion protein as described before (15). Briefly, the plasmid was transformed into *Escherichia coli* BL21, and protein expression was induced by 1 mM isopropyl 1-thio-β-D-galactopyranoside (Roth) for 4 h. After bacterial lysis in 3% Sarkosyl buffer, the RAP-GST protein was pulled down with glutathione-Sepharose beads (Amershams Biosciences), eluted with a 10 mM glutathione, 50 mM Tris solution, and dialyzed against phosphate-buffered saline overnight.

**Antibodies**—Primary antibodies used were monoclonal phospho-p44/42 MAPK (E10), polyclonal p44/42 MAP kinase (both from Cell Signaling, Danvers, MA), monoclonal ANTI-FLAG® M2 antibody, and polyclonal actin 20-33 antibody (both from Sigma). The polyclonal LRP1 antibody (1704), which reacts with the cytoplasmic domain of LRP1, has been described previously (16). Secondary goat antibodies against mouse and rabbit were both horseradish peroxidase-coupled and purchased from The Jackson Laboratory.

**Tissue Culture**—The immortalized cell line HT22, a subclone of the HT4 cell line, was derived from the mouse hippocampus and therefore has neuronal properties (17). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 mM minimal essential medium sodium pyruvate (all from Invitrogen), and 100 units/ml penicillin and 100 μg/ml streptomycin (both from Cambrex) in a humidified 5% CO2 incubator at 37 °C.

Hippocampal neurons were prepared from either Sprague-Dawley rat embryos at embryonic day 17 or 18 or C57Bl6 wild-type and C57Bl6/129 LRP1 knock-in mouse embryos at embryonic day 15 or 16 (18). In brief, hippocampi were collected in Hanks’ balanced saline solution (Invitrogen), cut in small pieces, and trypsinized (0.05% trypsin, 0.02% EDTA in phosphate-buffered saline) for 15 min. After mechanical dissociation in Neurobasal (Invitrogen) containing B-27 and 2-fold Glutamax (both from Invitrogen), cells were centrifuged at 500 × g for 4 min and resuspended again in Neurobasal for counting. The cells were then plated onto poly-L-ornithine (100 μg/ml; Sigma)-coated 6-well plates at a density of 5 × 105 cells/well. For calcium-imaging experiments, cells were plated onto poly-L-ornithine-coated glass coverslips in 4-cm dishes. The medium was changed the next day, and the cells were cultured for another 14 days at 37 °C in a humidified 5% CO2 incubator.

**Drug Treatment**—HT22 cells were grown to 80% confluence and serum-starved for 24 h in serum-free Dulbecco’s modified Eagle’s medium before tPA treatment. tPA (Actilyse) was reconstituted in double-distilled H2O and added to the culture medium for 30 min or 1 h at 37 °C, respectively. Stimulation of MAPK with stPA was detectable at a concentration of 10 μg/ml, whereas 40 μg/ml Actilyse was required to induce the same degree of MAPK stimulation. Therefore, we concluded that 10 μg/ml stPA functionally equals 40 μg/ml Actilyse, which we from now on refer to as tPA. The inhibitors MK-801 (10 μM) and U0126 (50 μM) were preincubated for 15 min, DL-AP5 (100 μM) was preincubated for 5 min, and RAP (500 nM) was preincubated for 1 h, and they were still present during tPA treatment. After 14 days in vitro (DIV), primary hippocampal neurons were treated as HT22 cells, except there was no medium change 24 h prior to stimulation.

**Calcium Phosphate-mediated Transfection of PSD95 and PDZ Domains**—HT22 cells were transfected with three different vector constructs corresponding to the PSD95 PDZ domain I, II, or III. Each single PDZ domain was cloned into the pcDNA 3.1 zeo (+) vector (Invitrogen), bearing a 3× FLAG sequence at its N terminus. The corresponding nucleotide sequences (rat) are as follows: PDZ1 (nucleotides 178–465), PDZII (nucleotides 463–747), and PDZIII (nucleotides 904–1206) (19). Additionally, a GW1-CMV-vector bearing the full-length PSD95 protein (a kind gift of Dr. Morgan Sheng) (20) was transfected. Cells were seeded in 6-well plates at ∼30% confluence and transfected with 2 μg of DNA/well via a calcium phosphate-mediated transfection protocol (21). 4–6 h post-transfection, the medium was changed to serum-free conditions for 24 h before cells were stimulated with tPA.

**LRP1 Knockdown with shRNA Vector Transfection**—The shRNA expression vector pALsh-LRP1 recognizes the 5’-region of the open reading frame of LRP1 (22). The generated self-annealing oligodeoxynucleotide targets the following sequence of the human LRP1 coding region: 5’-TAA GAC TTT CAG CCCCAA GCA GTT-3’ (position 72–95 of the human LRP1 open reading frame). pALsh vector alone served as transfection control. The mouse neuronal cell line HT22 was grown in 6-well plates until ∼50% confluence and transfected using Lipofectamine™ 2000 reagent (Invitrogen) at a ratio of 2 μg of plasmid DNA to 4 μl of Lipofectamine. To obtain better transfection efficiency, the cells were retransfected after 48 h with the same amount of DNA for another 24 h in serum-free Dulbecco’s modified Eagle’s medium. After 72 h in total, cells were stimulated with tPA, lysed, subjected to 10 or 12% SDS-PAGE,
LRP1-mediated NMDA Receptor Activation

A

- tPA
+ tPA +
P-Erk1/2
Erk1/2

30min
1h
primary neurons

B

- tPA
+ tPA +
P-Erk1/2
Erk1/2

30min
1h
HT22

C

- tPA
+ tPA +
P-Erk1/2
Erk1/2

HT22

FIGURE 1. tPA activates the Erk1/2 kinase in primary hippocampal neurons and in the neuronal cell line HT22. A, Western blot analysis of E18 rat hippocampal neurons. Total lysate was probed with monoclonal anti-phospho-Erk1/2 or total Erk1/2 antibody after treatment with tPA for 30 min or 1 h. Water (−) and 10% fetal bovine serum (+) served as negative and positive controls, respectively. B, comparable treatment of HT22 cells with tPA. C, Western blot analysis of HT22 cell lysate showing the diminished Erk1/2 phosphorylation after preincubation with 10 μM MEK inhibitor U0126 for 15 min and subsequent tPA treatment. DMSO and U0126 alone served as negative controls. Immunoblots shown are representative examples of the results from at least three independent experiments.

and tested for LRP1 knockdown and Erk1/2 activation with Western blotting as described below.

siRNA Knockdown—Stealth™/siRNA duplex oligoribonucleotides against mouse PSD95, Shc1, and FE65 were purchased from Invitrogen, carrying the following sequences: 5′-GGA GUA UGA GGA GAU CAC AUU GGA A-3′ (PSD95), 5′-CCA GAU GCU CAA GUG CCA CGU GGU U-3′ (FE65), and 5′-GGC UGU GUG GAG GUC UUA CAG UCA A-3′ (Shc1). Transfections in primary hippocampal neurons or HT22 cells were carried out with 40 nM (PSD95), 20 nM (Shc1), and 100 nM (FE65) siRNA duplex with LipofectamineTM 2000 reagent (Invitrogen) for 48 h.

SDS-PAGE and Western Blot Analysis—Primary hippocampal cultures as well as HT22 cells were lysed in radioimmune precipitation buffer (50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing proteinase inhibitors (complete; Roche Applied Science) and phosphatase inhibitors (1 mM sodium orthovanadate (Sigma) and 10 mM sodium fluoride (AppliChem)). Protein concentration was determined by the BCA method (Pierce), and equal amounts of total protein (+/−) served as negative and positive controls, respectively. B, comparable treatment of HT22 cells with tPA. C, Western blot analysis of HT22 cell lysate showing the diminished Erk1/2 phosphorylation after preincubation with 10 μM MEK inhibitor U0126 for 15 min and subsequent tPA treatment. DMSO and U0126 alone served as negative controls. Immunoblots shown are representative examples of the results from at least three independent experiments.

Calcium Imaging—The fluorescent calcium indicator calcium green was used to analyze changes of the [Ca2+]i in cultured neurons and HT22 cells. In detail, after 24 h in serum-free medium, cells cultured on glass coverslips were loaded (60 min at 37 °C) with the fluorescent dye by adding 10 μM calcium green in pluronic acid (both from Molecular Probes, Leiden, Netherlands) to the culture medium. After loading, excess dye was removed, and the cells were kept in Hanks’ balanced saline solution (PAA, Linz, Austria) supplemented with 2 mM CaCl2, 1 mM MgCl2, and 10 mM glucose and transferred to a temperature-controlled recording chamber adapted to an upright microscope (BX51WI, Olympus, Hamburg, Germany), equipped with a Nipkow spinning disk confocal system (QLC10; Visi-tech, Sunderland, UK) and a krypton/argon laser (Laser Physics, Cheshire, UK). The fluorescence of calcium green was excited at 488 nm. After background subtraction, changes of the emitted fluorescence were analyzed using Metamorph imaging software (Molecular Devices Corp, Downingtown, PA). Changes of [Ca2+]i in individual cell somata are shown as relative changes of calcium green fluorescence compared with the starting fluorescence intensity (ΔF/ΔF0). The addition of tPA (40 μg/ml) was conducted 120 s after the recording was started. Inhibitors (10 μM MK-801 or 500 nM RAP) were preincubated for at least 30 min and were present during the whole recording time.

In another set of experiments, calcium green was used to determine [Ca2+]i of HT22 cells or primary hippocampal neurons cultured in 96-well plates, and fluorescence intensity was quantified using a fluorescence plate reader (Infinite F200; Tecan, Salzburg, Austria). Dye loading and pretreatment with inhibitors was performed as mentioned above. Fluorescence was excited at 485 nm, and the emitted light was detected at 535 nm. The maximum increase of calcium green fluorescence during a time window of 5 min after the start of the stimulation was normalized to the fluorescence intensity prior to stimulation (ΔF/ΔF0max).

Glutamate Measurement—Release of L-glutamate from HT22 cells in response to tPA stimulation was examined using an Amplex Red kit (Molecular Probes, Leiden, Netherlands). Cells were cultured in 96-well plates at a density of 16,000 cells/well. 24 h after seeding, culture medium was changed to serum-free medium for another 24 h. Afterward, HT22 cells were incubated for 1 or 5 min with 40 μg/ml tPA in Hanks’ balanced saline solution with Ca2+, Mg2+ and glucose. 50-μl samples were used in the assay according to the manufacturer’s instructions. The resulting fluorescence was measured using a fluorescence microplate reader (Infinite F200; Tecan). Fluorescence was excited at 540 nm, and the emission was detected at 580 nm.

Reverse Transcription-PCR—Total RNA was isolated from either HT22 cells or primary hippocampal neurons (RNeasy minikit; Qiagen). Reverse transcription-PCRs (One-Step reverse transcription-PCR kit; Qiagen) were carried out in 50 μl of reaction solution containing 500 ng of total RNA, 0.4 mM deoxynucleotides, 0.6 μM forward and reverse primer, 5 units of reverse transcriptionase, and 200 units of reverse transcriptase (Promega). The reverse transcription reaction was performed at 42 °C for 60 min, followed by 95 °C for 5 min. The PCR reaction was conducted using a One-Step RT-PCR kit (Qiagen) following the manufacturer’s instructions. The resulting fluorescence was measured using a fluorescence plate reader (Infinite F200; Tecan). Fluorescence was excited at 540 nm, and the emission was detected at 580 nm.
LRP1-mediated NMDA Receptor Activation

FIGURE 2. RAP acts as a functional inhibitor of tPA-mediated Erk1/2 activation. A, Erk1/2 phosphorylation of HT22 cells treated with 500 nM RAP 1 h prior to tPA stimulation is strongly inhibited compared with control treatment. Reprobing with polyclonal 1704 anti-LRP1 antibody revealed no alteration in LRP1 processing (LRP1 β-chain, 85 kDa; LRP1 C-terminal fragments, 18 kDa) after tPA treatment. B, stimulation of embryonic day 18 rat hippocampal neurons with tPA following a 1-h preincubation with 500 nM RAP abolished phosphorylation. Immunoblots shown are representative examples of the results from at least three independent experiments.

FIGURE 3. Knockdown of LRP1 protein with short hairpin LRP1 plasmid (shLRP1) results in decreased Erk1/2 phosphorylation. A, Western blot analysis of reduced LRP1 β-chain (85 kDa) expression after 72-h transfection with short hairpin LRP1 plasmid or vector control before tPA treatment in HT22 cells. B, quantification of short hairpin LRP1 plasmid β-chain knockdown normalized to actin illustrates a reduction in protein expression by ~70% (*, p < 0.05 versus vector, n = 3). C, prominent decrease in Erk1/2 phosphorylation after tPA stimulation in shLRP1 knockdown (72 h) compared with vector control. Note that after tPA stimulation, the Erk1/2 phosphorylation in shLRP1-treated cells is almost completely abolished. Immunoblots shown are representative examples of the results from at least three independent experiments.

 RNase inhibitor, and 2 μl of enzyme mixture. The reaction solution was incubated at 50 °C for 30 min and denatured at 94 °C for 15 min, followed by 45 cycles at 94 °C for 1 min, 53 °C for 30 s, and 72 °C for 2 min. The final extension step was at 72 °C for 10 min. The primers were as follows: forward primer for NMDA receptor subunit 1 (mouse, gi: 6680094) was 5′-AGT GCT GTT ATG GCT TCT GC-3′ (exon 10, positions 1451–1470); reverse primer was 5′-TCG GCC AAA GGG ACT GAA GC-3′ (exon 13, positions 1857–1838). The amplified products (20 μl) were separated by 2% agarose gel and visualized by ethidium bromide staining.

Data Analysis—Results were expressed as mean values ± S.E. Statistically significant effects of the calcium-imaging experiments were assessed by two-way analyses of variance followed by post hoc Tukey test for multiple comparisons, and a value of p < 0.05 was considered to indicate statistical significance of the results.

RESULTS

Specific Activation of Erk1/2 Kinases by tPA in Neuronal Cells—Previous studies from Medina et al. (11) and Nicole et al. (10) have demonstrated that tPA stimulates the MAPK pathway and NMDA receptor-mediated calcium influx into N2a cells and hippocampal neurons. Although both groups postulated a tPA-mediated activation of the NMDA receptor, they proposed different mechanisms of tPA-mediated downstream signaling. Therefore, we set out to determine the tPA-mediated effects using two related model systems: the mouse neuronal hippocampus-derived cell line HT22 (17) and rat primary hippocampal neurons. As illustrated in Fig. 1, tPA (Actilyse) is able to induce the phosphorylation of Erk1/2 without altering the total amount of the protein, both in primary hippocampal cells (Fig. 1A) and HT22 cells (Fig. 1B). The amount of Actilyse used in our experiments corresponds to 10 μg/ml pure synthetic tPA (data not shown). Maximal Erk1/2 activation in the two cell systems was observed between 30 min and 1 h, respectively, after the start of stimulation. Shorter stimulations starting from 2 to 15 min did not induce a significant increase in phospho-Erk1/2 in our cell systems and were hence thought to be inappropriate for further studies (data not shown).

To further examine the pathway leading to the activation of Erk1/2, we preincubated the cells for 15 min with U0126, an inhibitor of the upstream MAPK kinase (MEK1/2). Subsequent tPA treatment resulted in no Erk1/2 phosphorylation compared with the normal tPA-treated cells, indicating that MEK1/2 is directly responsible for this effect in HT22 cells (Fig. 1C).

RAP, an Antagonist for Lipoprotein Receptor Ligand Binding Blocks tPA-mediated Erk1/2 Activation—It has been previously suggested that LRPI mediates tPA-induced Erk1/2 phosphorylation (6). To verify the involvement of LRPI in tPA-mediated signaling, we incubated neuronal cells with RAP, thereby inhibiting all ligands from binding to lipoprotein receptors. Preincubation of 500 nM RAP-GST 1 h prior to tPA stimulation resulted in a significant decrease in phospho-Erk1/2 in both primary hippocampal neurons and HT22 cells (Fig. 2A). This effect was confirmed by Western blot analysis (Fig. 2B), indicating that RAP acts as a functional inhibitor of tPA-mediated Erk1/2 activation.

LRP1-mediated NMDA Receptor Activation

FIGURE 2. RAP acts as a functional inhibitor of tPA-mediated Erk1/2 activation. A, Erk1/2 phosphorylation of HT22 cells treated with 500 nM RAP 1 h prior to tPA stimulation is strongly inhibited compared with control treatment. Reprobing with polyclonal 1704 anti-LRP1 antibody revealed no alteration in LRP1 processing (LRP1 β-chain, 85 kDa; LRP1 C-terminal fragments, 18 kDa) after tPA treatment. B, stimulation of embryonic day 18 rat hippocampal neurons with tPA following a 1-h preincubation with 500 nM RAP abolished phosphorylation. Immunoblots shown are representative examples of the results from at least three independent experiments.

FIGURE 3. Knockdown of LRP1 protein with short hairpin LRP1 plasmid (shLRP1) results in decreased Erk1/2 phosphorylation. A, Western blot analysis of reduced LRP1 β-chain (85 kDa) expression after 72-h transfection with short hairpin LRP1 plasmid or vector control before tPA treatment in HT22 cells. B, quantification of short hairpin LRP1 plasmid β-chain knockdown normalized to actin illustrates a reduction in protein expression by ~70% (*, p < 0.05 versus vector, n = 3). C, prominent decrease in Erk1/2 phosphorylation after tPA stimulation in shLRP1 knockdown (72 h) compared with vector control. Note that after tPA stimulation, the Erk1/2 phosphorylation in shLRP1-treated cells is almost completely abolished. Immunoblots shown are representative examples of the results from at least three independent experiments.
LRP1-mediated NMDA Receptor Activation

A

- IPA + MK-801 + IPA

P-Erk1/2 Erk1/2

30 min HT22

B

- IPA + RAP + IPA

P-Erk1/2 Erk1/2

30 min primary neurons

C

- IPA + DL-AP5 + IPA

P-Erk1/2 Erk1/2

30 min HT22

D

- IPA + DL-AP5 + IPA

P-Erk1/2 Erk1/2

30 min primary neurons

E

- IPA + D-IPA

P-Erk1/2 Erk1/2

HT22

FIGURE 4. MK-801 and DL-AP5 prevent tPA-induced Erk1/2 phosphorylation. A, preincubation of HT22 cells with the NMDA receptor open channel blocker MK-801 (10 µM) for 15 min and subsequent tPA stimulation result in strongly reduced Erk1/2 phosphorylation. In contrast, MK-801-nontreated cells reveal increased Erk1/2 phosphorylation after tPA stimulation, as shown before. B, RAP (500 nM) and MK-801 (10 µM) pretreatment of primary hippocampal rat embryonic neurons result in similar reduction in Erk1/2 phosphorylation after tPA treatment. Note that an inhibitor for Lipoprotein receptor binding and an NMDA receptor blocker resulted in similar reduction in Erk1/2 phosphorylation. C, preincubation of HT22 cells with 100 µM DL-AP5, a competitive NMDA receptor antagonist, for 5 min results in dramatic reduction of Erk1/2 phosphorylation after tPA stimulation. 0.2 mM NaOH served as negative solvent control for DL-AP5. D, DL-AP5-lowered Erk1/2 phosphorylation after tPA treatment is also detected in rat primary hippocampal neurons. E, HT22 cells incubated with dialyzed tPA (D-tPA) show similar effects on Erk1/2 phosphorylation as with nondialyzed tPA. Immunoblots shown are representative examples of the results from at least three independent experiments.

in a pronounced decrease of Erk1/2 phosphorylation compared with total Erk1/2 protein in HT22 cells (Fig. 2A, upper bands). Similar effects were detected for primary neurons (Fig. 2B). To investigate a possible proteolytic influence of tPA on LRP1, we measured the protein level of LRP1 β-chain (85 kDa) and C-terminal fragments (~18 kDa) using the anti-LRP1 polyclonal 1704 antibody (16). As shown in Fig. 2A (lower bands), tPA treatment did not alter LRP1 protein expression or processing, since no differences in LRP1 β-chain or LRP1 C-terminal fragment protein levels could be detected in tPA-treated compared with untreated control cells.

Specific Knockdown of LRP1 with a Short Hairpin RNA Plasmid Construct Results in Decreased Erk1/2 Phosphorylation—To address the question of whether the observed RAP effect on Erk1/2 phosphorylation is actually due to a blockade of LRP1 and not due to any other low density lipoprotein receptor family member, we employed a well documented vector-based shRNA knockdown strategy to specifically block LRP1 protein translation in HT22 cells (22). As presented in Fig. 3A, Western blot analysis of the short hairpin LRP1 plasmid (shLRP1)-transfected cells confirms the down-regulation of LRP1 β-chain in contrast to the pALsh vector control after 72 h of transfection. The quantitative data on the protein knockdown (Fig. 3B) demonstrate a reduction of LRP1 β-chain by ~70%. Subsequent tPA stimulation resulted in an almost abolished Erk1/2 phosphorylation signal in the shRNA-transfected cells (Fig. 3C). Taken together, these data indicate that LRP1 is specifically responsible for the tPA-induced Erk1/2 activation and therefore mediates the signal from the cell surface into the cell.

NMDA Receptor Open Channel Blocker MK-801 Prevents tPA-induced Erk1/2 Phosphorylation—It has been proposed that tPA might transduce its signal into the cell via direct binding to the NMDA receptor on the cell surface (10, 23, 24). Therefore, we wanted to investigate the role of this receptor in our model system by blocking calcium influx with MK-801, a noncompetitive NMDA receptor inhibitor. Consistent with previous findings (11), tPA-induced Erk1/2 phosphorylation in primary neurons and HT22 cells was reduced after preincubation with 10 µM MK-801 (Fig. 4, A and B). Most interestingly, MK-801 reduced the tPA-mediated Erk1/2 phosphorylation in primary hippocampal neurons to similar levels as seen with RAP (Fig. 4B), suggesting that NMDA receptors and LRP1 either act in parallel or in succession in this signaling cascade.

DL-AP5, a competitive NMDA receptor antagonist, corroborates the involvement of NMDA receptors in tPA-mediated Erk1/2 activation. To further study the role of the NMDA receptor, both cell types were additionally preincubated with the competitive NMDA receptor antagonist DL-AP5. As expected, the tPA-induced Erk1/2 activation was abolished both in HT22 cells (Fig. 4C) and primary neurons (Fig. 4D), further proving the involvement of NMDA receptors in LRP1-mediated tPA signaling. For control purposes, cells were co-incubated with 0.2 mM NaOH and tPA to rule out any solvent effect of DL-AP5 on Erk1/2 phosphorylation.

As we used the well established drug Actilyse (Boehringer Ingelheim) instead of pure synthetic tPA, we wanted to rule out any effects of possible low molecular weight contaminants like, for example, glutamate, which would directly activate NMDA receptors, or L-arginine as a prerequisite for NO generation, which could lead to stimulation of MAPK (25). Therefore, we dialyzed the Actilyse against double-distilled H2O with three buffer changes. The subsequent stimulation of HT22 cells with
LRP1-mediated NMDA Receptor Activation

To verify the existence of functional NMDA receptors in HT22 cells, these cells were treated with NMDA or glutamate. Subsequently, calcium influx was assayed in 96-well plates with a fluorescence plate reader. As shown in Fig. 6A, Actilyse induced a significant increase of [Ca$^{2+}$], that was equal to the effect of pure stPA (both *, $p < 0.05$ versus control, $n = 4$). Interestingly, NMDA and glutamate treatment led to a comparable increase in intracellular calcium levels, which could be reduced by preincubation with the specific NMDA receptor inhibitor MK-801, clearly demonstrating the existence of functional NMDA receptors in HT22 cells (#, $p < 0.05$, $n = 4$).

Additionally, we tested for the mRNA expression level of NMDA receptor subunit 1 in HT22 cells compared with primary hippocampal neurons (Fig. 5, A and C) and cultured HT22 cells (Fig. 5, B and D). The pattern of the calcium signal was similar in both cell types, showing an initial peak and a subsequent plateau phase. This calcium response to tPA was abolished if the cells were pretreated with an NMDA receptor antagonist MK-801 (Fig. 5, A and B) or the LRP1 binding inhibitor RAP (Fig. 5, C and D). For statistical evaluation, we compared the maximum calcium increase after the addition of tPA. As shown in Fig. 5E, the calcium signal evoked by tPA was significantly reduced by MK-801 or RAP treatment (* or #, $p < 0.05$, $n = 43–91$ single cells from at least three individual coverslips). These experiments suggest that tPA-LRP1 signaling can activate NMDA receptors within several seconds of tPA application. Since these calcium measurements were performed in fresh buffer rather than in conditioned medium, which could contain trace amounts of released glutamate, it is unlikely that glutamate participated in activating NMDA receptors.

However, to completely exclude the possibility that the tPA-induced calcium increase in HT22 cells was due to a release of endogenous glutamate, glutamate concentrations after tPA treatment were measured in the supernatant using an Amplex Red glutamate assay (Molecular Probes). Importantly, tPA stimulation of HT22 did not reveal a detectable glutamate release in the cultures compared with vehicle-treated controls (Fig. 5F). Thus, tPA-induced glutamate release, which then activates NMDA receptors, can be excluded in these experiments.

The Functional Cooperation of LRP1 and NMDA Receptor Can Be Certified by Calcium-imaging Experiments—To provide further evidence for an LRP1-mediated effect on NMDA receptor activation, we measured calcium influx into the cells in response to tPA stimulation. The addition of tPA resulted in a rapid increase of [Ca$^{2+}$], in both HT22 cells (Fig. 5, A and C) and primary hippocampal neurons (Fig. 5, B and D). The pattern of the dialyzed tPA resulted in an Erk1/2 phosphorylation comparable with that seen without dialysis (Fig. 4E). This implies that the observed Erk1/2 activation through the NMDA receptor pathway is due to tPA itself and not to any other component in the tPA solution. These results strongly suggest a pivotal role of tPA in LRP1-mediated NMDA receptor activation.

FIGURE 5. tPA-induced calcium signaling in rat primary hippocampal neurons and HT22 cells. The calcium indicator calcium green was used to analyze tPA-induced changes of [Ca$^{2+}$] in HT22 cells (A and C) or cultured primary neurons (B and D) using confocal laser-scanning microscopy (20× magnification). In A–D, each trace represents the single cell measurement of changes in soma [Ca$^{2+}$] in response to tPA. The time courses of [Ca$^{2+}$] of three representative cells from the same field of view are shown for each condition. Measurements from cells pretreated with MK-801 (10 μM; A and B) or RAP (500 nM; C and D) are shown in gray. Representative matched positive controls from the same cultures, respectively, are shown in black (A–D). E, average data of calcium responses from all measurements; the maximum calcium response after the tPA addition was significantly reduced by MK-801 or RAP (* or #, $p < 0.05$, $n = 43–91$ single cells from at least three individual coverslips). These experiments suggest that tPA-LRP1 signaling can activate NMDA receptors within several seconds of tPA application. Since these calcium measurements were performed in fresh buffer rather than in conditioned medium, which could contain trace amounts of released glutamate, it is unlikely that glutamate participated in activating NMDA receptors.

However, to completely exclude the possibility that the tPA-induced calcium increase in HT22 cells was due to a release of endogenous glutamate, glutamate concentrations after tPA treatment were measured in the supernatant using an Amplex Red glutamate assay (Molecular Probes). Importantly, tPA stimulation of HT22 did not reveal a detectable glutamate release in the cultures compared with vehicle-treated controls (Fig. 5F). Thus, tPA-induced glutamate release, which then activates NMDA receptors, can be excluded in these experiments.
LRP1-mediated NMDA Receptor Activation

A

FIGURE 6. HT22 cells express functional NMDA receptors. A, intracellular Ca\(^{2+}\) levels were analyzed in HT22 cells using the fluorescence indicator calcium green. Dye-loaded cells were stimulated with NMDA (30 \(\mu\)M), glutamate (1 mM), Actilyse, or stPA. MK-801 pretreatment was performed 30 min before NMDA or glutamate stimulation. Fluorescence intensities are given as maximal changes of calcium green fluorescence in relation to the starting fluorescence ([F/F]\text{max}, * p < 0.05 versus control; #, p < 0.05 versus NMDA/glutamate; n = 4). B, reverse transcription-PCR for NMDA receptor subunit 1 of 400 ng of mRNA extracted from HT22 cells gives the same cDNA signal as for primary neurons. H\(_2\)O and a sample without reverse transcriptase (−RT), respectively, served as negative controls. Experiments were performed in triplicate, and one representative experiment is shown.

B

mary hippocampal neurons by reverse transcription-PCR. As shown in Fig. 6B, mRNA extracted from HT22 cells generated a specific cDNA signal of ~400 bp (expected 407 bp) corresponding to that of primary neurons, whereas two negative controls did not show any band. Taken together, we conclude that HT22 cells do in fact express functional NMDA receptors.

PSD95 Overexpression or Knockdown Interrupts the LRP1-NMDA Receptor Cross-talk—To tackle the mechanism leading to the LRP1-mediated NMDA receptor activation, we hypothesized that a linker molecule might bridge the intracellular C-terminal portions of LRP1 and the NMDA receptor. As has been shown before, PSD95 can act as an intracellular linker between LRP1 and the NMDA receptor (4, 26). If PSD95 or any other adaptor protein might link both proteins together, one should be able to interrupt the LRP1-mediated signal to the NMDA receptor by overexpressing a truncated binding partner still binding to LRP1 but lacking the ability to bind to the NMDA receptor in parallel. Therefore, three truncated FLAG-tagged PSD95 constructs were generated, bearing the single PDZ domains (Fig. 7A). Only overexpression of PSD95 PDZ domain III resulted in a strong reduction in Erk1/2 phosphorylation after tPA treatment compared with the mock control and the other two PDZ domains I and II (Fig. 7B). The quantification shows a 70% reduction in Erk1/2 phosphorylation in cells transfected with PDZIII compared with mock-transfected neurons (Fig. 7C). This lets us hypothesize that the overexpressed PDZIII domain might displace either 1) the endogenous PSD95 or 2) any other adaptor protein from its binding site at the LRP1 C terminus and thereby prevent the bridging of an LRP1-adaptor protein-NMDA receptor complex, which might be a prerequisite for tPA-mediated NMDA receptor activation and subsequent MAPK signaling. By overexpression of PSD95 protein, one might expect an increase in Erk1/2 activation due to enhanced linkage between the two receptors. To test this hypothesis, the full-length PSD95 protein was overexpressed in HT22 cells, and interestingly, a decrease in the phosphorylation of Erk1/2 was observed compared with control conditions (Fig. 7D). We interpret this result by indicating that overexpression of intact PSD95 must have sequestered endogenous LRP1 away from the NMDA receptor, thus producing a phenotype similar to overexpression of truncated PSD95 constructs. A similar effect could be observed with a specific siRNA knockdown of PSD95 before tPA treatment, where reduction in the protein level might also lead to less linkage and therefore less Erk1/2 activation (Fig. 7E). To rule out the involvement of any other known adaptor protein for LRP1 like Shc1 or FE65 (27, 28) in tPA-mediated Erk1/2 activation, we made use of small interfering RNAs to knock down Shc1 and FE65 protein levels. Subsequent tPA stimulation did not lead to a decreased Erk1/2 activation, suggesting no functional role of these two adaptor proteins in our model system (supplemental Fig. S1).

The Distal NPXY Domain of LRP1 Might Be Responsible for the NMDA Receptor-mediated Intracellular Signal Transduction—To narrow down the binding site in LRP1 for such an adaptor protein, which might additionally interact with the NMDA receptor complex, we used a LRP1 knock-in strategy. In a recent study, mutant LRP1 knock-in mouse models were generated using recombinase-mediated cassette exchange technology (18). Mutations were introduced into either the first (NPTY → AATA) or the second NPXY motif (NPYYATL → AAVATL) of the LRP1 intracellular domain (LRP1-ICD). The homozygous embryos from the proximal NPXY knock-in mice have a lethal phenotype and die perinatally, whereas homozygous mice with an inactivation of the distal NPXY are normal and fertile. It has been demonstrated by several laboratories that the distal NPXY motif (NPXY(2)) in LRP1 interacts with many different cytosolic adaptor proteins (27, 28). In this regard, the primary hippocampal neurons derived from these NPXY(2) knock-in mice were used to prove the link of LRP1 to the NMDA receptor. Indeed, tPA stimulation of primary hippocampal neurons from embryonic day 15 NPXY(2) embryos shows a prominent decrease in Erk1/2 phosphorylation (Fig. 8B) compared with the wild-type neurons (Fig. 8A). These Western blot data could be confirmed by calcium-imaging experiments in the same primary neurons mentioned before, since the NPXY(2) knock-in cells also led to a decreased calcium influx after tPA treatment compared with the wild-type cells (Fig. 8C; *, p < 0.05 versus wild type tPA, n = 4). These results clearly demonstrate the importance of a functional LRP1 C terminus in tPA-mediated activation of NMDA receptors and subsequent Erk1/2 phosphorylation.

DISCUSSION

Recently, it has been suggested that the NMDA receptor might act as the receptor for tPA binding and facilitate tPA-mediated downstream signaling in neuronal systems (10). Two
contradictory mechanisms have been suggested to explain this effect; one involves proteolytic cleavage of the NMDA receptor, and the other one is independent of NMDA receptor cleavage upon tPA binding (10, 11). However, other studies have implicated LRP1 as the major tPA receptor in the brain facilitating a subsequent downstream signaling (5, 6). Despite overall agreement on the stimulatory role of tPA on neuronal cells, the mechanism behind this function remains controversial. The current study was designed to address the question of how tPA mediates its downstream signaling and which cell surface receptors bind tPA and possibly transmit a signal into the cell. Our study provides strong evidence that LRP1 acts as the main tPA-binding receptor in neuronal cultures and that the resulting LRP1-tPA complex stimulates calcium influx via gating of NMDA receptors through the adaptor protein PSD95.

FIGURE 7. PSD95 as potential linker protein mediates tPA-induced Erk1/2 activation. A, schematic illustration of the generated PSD95 PDZ domains. The indicated numbers correspond to the amino acids of the rat PSD95 sequence. B, overexpression of PSD95 PDZ domain III in HT22 cells results in diminished Erk1/2 phosphorylation after tPA treatment compared with the other two domains and the mock control (pcDNA 3.1). The corresponding FLAG blot shows the expression level of all three constructs. C, quantification of the PDZIII effect shows a 70% reduction in Erk1/2 phosphorylation normalized to total Erk1/2 levels (*, p < 0.05 versus mock, n = 3). D, overexpression of a full-length PSD95 vector construct results in reduced Erk1/2 activation after tPA treatment compared with control transfection. E, siRNA knockdown of PSD95 (40 nM) reduces tPA-induced Erk1/2 activation. Immunoblots shown are representative examples of the results from at least three independent experiments.
LRP1-mediated NMDA Receptor Activation

**FIGURE 8.** The distal NPXY motif in the intracellular C terminus of LRP1 is important for NMDA receptor-mediated signal transduction. A, primary hippocampal neurons derived from mice embryos bearing the LRP1 wild-type allele show an increase in Erk1/2 phosphorylation after tPA stimulation. B, corresponding neurons from mutant NPXY(2) embryos show no increase in Erk1/2 phosphorylation compared with the negative control. Immunoblots shown are representative examples of the results from at least three independent experiments. C, intracellular calcium levels were analyzed in primary neurons mentioned above using the fluorescence indicator calcium green. Dye-loaded wild-type or mutant cells were stimulated with tPA or control agent. Fluorescence intensities are given as maximal changes of calcium green fluorescence in relation to the starting fluorescence ([F0/F0]max). *p < 0.05 versus wild-type tPA; n = 4. Note that the second (distal) NPXY(2) domain of LRP1 plays a crucial role in tPA-mediated activation of the NMDA receptor.

tPA-mediated stimulation of the downstream Erk1/2 phosphorylation. Additionally, we could show that an excess supply of full-length PSD95 protein also leads to a disrupted complex, most probably by sequestering LRP1 away from NMDA receptors. Here we hypothesize that overexpression of PSD95 results in increased binding of PSD95 to each receptor, thereby impeding the bridging capacity of PSD95 itself. This clearly demonstrates that prevention of endogenous adaptor protein binding to the C terminus of LRP1 might disrupt the LRP1-NMDA receptor complex formation, resulting in a reduced downstream signaling.

If indeed LRP1 would be the primary target for tPA binding before transferring the signal onto the NMDA receptor, one would predict that mutations in the LRP1 C-terminal binding site for most intracellular ligands would abolish tPA-mediated signaling to the NMDA receptor. LRP1 carries two C-terminal NPXY motifs that have been shown to interact with adaptor proteins; however, most protein interactions occur through the second NPXY motif of LRP1 (30, 32, 33). Therefore we made use of LRP1 knock-in mutant mice, where the distal NPVYATL site was mutated to AAVAATL in the endogenous LRP1 gene, preventing any adaptor protein binding (18). Primary neurons derived from these mice showed neither any tPA-mediated Erk1/2 stimulation nor increased calcium influx compared with control neurons carrying the wild-type LRP1 sequence. This suggests that the signaling to the NMDA receptor might be mediated through intracellular adaptor proteins binding to the distal NPXY motif. By using siRNA knock-down experiments for three potential LRP1 adaptor protein candidates, PSD95, FE65, and Shc1, we were specifically able to identify PSD95 as a linker protein connecting the C terminus of LRP1 to the NMDA receptor and thereby mediating the signal. At this point, we cannot exclude the possibility that, after tPA binding to LRP1 and recruiting adaptor proteins for bridging LRP1 to the NMDA receptor intracellularly, there might also be a subsequent conformational change of the complex leading to extracellular interaction of the tPA-LRP1 complex with the NMDA receptor that might stimulate the calcium channel. However, we provide strong evidence for a tripartite LRP1-PSD95-NMDA complex as a starting point in tPA-mediated signaling events. As has been implicated before, this tPA-stimulated Erk1/2 activation might have detrimental neurotoxic effects on neurons. It has been speculated that tPA might be involved in Alzheimer disease. The amyloid-β peptide derived from the amyloid precursor protein might be capable of increasing tPA production, thereby leading to NMDA recep-
tor-mediated apoptosis and necrosis via GSK3 activation and tau hyperphosphorylation (11). Further concerns come from studies showing the ability of tPA to induce MMP-9 gene expression via LRPI, promoting neuronal cell death by degradation of extracellular matrix components (6, 34). These neurotoxic side effects are especially important, since tPA is used as a thrombolytic agent approved by the United States Food and Drug Administration for acute stroke therapy (35). In conclusion, our results provide detailed insights into unraveling the mechanism of tPA-mediated signaling events. We suggest that tPA binding to LRPI is a prerequisite for NMDA receptor activation and downstream signaling events, which are mediated via the distal NPY domain and the adaptor protein PSD95 (Fig. 9). These findings indicate that LRPI, PSD95, and NMDA receptor act as a tripartite complex in tPA-mediated signaling events, giving LRPI a more important role in neuronal pathophysiological function than has been previously suggested.

Acknowledgments—We thank C. Behl for providing the HT22 cell line and M. Plenikowski for designing the graphs.

REFERENCES