Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 481 (2003) 147–151
www.elsevier.com/locate/ejphar

Immunohistochemical detection of the calcitonin receptor-like receptor protein in the microvasculature of rat endothelium

Stefanie Hagner[a,*], Rainer Haberberger[b], Deborah L. Hay[c], Paul Facer[d], Katja Reiners[b], Karlheinz Voigt[a], Gerard P. McGregor[a]

[a] *Institute of Physiology, University of Marburg, Deutschhaus Strasse 2, D-35037 Marburg, Germany*
[b] *Institute of Anatomy and Cell Biology, University of Giessen, Giessen, Germany*
[c] *Peripheral Neuropathy Unit, Imperial College Medical School, London W12 ONN, UK*
[d] *School of Life Sciences, Aston University, Birmingham B4 7ET, UK*

Received 19 May 2003; received in revised form 4 September 2003; accepted 10 September 2003

Abstract

Calcitonin-gene-related peptide and adrenomedullin have similar and potent vascular effects, which appear to be mediated by the G protein-coupled calcitonin receptor-like (CRL) receptor. Using immunohistochemical and Western blot analyses, we have obtained novel evidence that CRL receptor is expressed in the rat vascular endothelium using an antibody to rat CRL receptor that we have raised and fully characterised. These results are an important basis for further studies aimed at determining the so far ill-defined functional significance of the extensive distribution of CRL receptor in the vascular endothelium.

Keywords: Calcitonin receptor-like receptor; CRL receptor; Vascular endothelium; Rat; Immunoreactivity

1. Introduction

Calcitonin gene-related peptide (CGRP) and adrenomedullin are structurally homologous peptides (Poyner et al., 2002) that exert similar biological effects. Both peptides have potent vasodilatory actions that appear to be mediated by a common G protein-associated receptor, the calcitonin receptor-like (CRL) receptor (Chu et al., 2001). CRL receptor has been cloned from several species (Born et al., 2002; Poyner et al., 2002) and acts either as a CGRP or an adrenomedullin receptor, depending with which receptor activity-modifying proteins (RAMPs) it is co-expressed (McLatchie et al., 1998). The CRL receptor/RAMP1 complex is the pharmacologically defined CGRP-1 receptor with which both CGRP and adrenomedullin interact, while RAMP2 or RAMP3 in combination with CRL receptor is an adrenomedullin receptor. The precise cellular localisation of CRL receptor in the rat is not yet determined due to the lack of an available and well-characterised antibody for precise immunochemical analysis. We aimed to develop and fully characterise an antibody for rat CRL receptor and to test whether the rat vascular endothelium also expresses the CRL receptor protein.

2. Materials and methods

2.1. Development and affinity-purification of rabbit polyclonal antibody

A rabbit polyclonal antiserum, MR003, to the carboxy (C)-terminus of rat CRL receptor sequence, SIQDIENVALKPEKLYDLVM (Chang et al., 1993) (Polypeptides Laboratories, Wolfenbuettel, Germany), was raised and affinity-purified according to a previously described scheme (Hagner et al., 2001).

2.2. Isolation and culture of rat pulmonary microvascular endothelial cells

Rat lung endothelial cells were prepared from adult Wistar rats by a previously described method (Duijvestijn et al., 1992) that employed a monoclonal antibody specific for rat endothelial cell antigen-1 (RECA-1). Wistar rats were

* Corresponding author. Tel.: +49-6421-2866649; fax: +49-6421-2862306.
E-mail address: stefanie-hagner@online.de (S. Hagner).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2003.09.030

sacrificed and lungs were prepared and minced before being incubated with magnetic beads [Dynabeads Pan Mouse Immunoglobulin (IgG), Dynal, Hamburg, Germany] coated with RECA-1 antibody (1 μg/10^7 beads). Endothelial cells bound to the beads were then recovered using a magnet before being resuspended in Endothelial Cell Growth Medium-MV (Promocell, Heidelberg, Germany). Cells were then cultivated for 15–18 days prior to RNA preparation or immunohistochemical analysis.

2.3. Stable transfection of human embryonal kidney (HEK) 293 cells

HEK 293 cells were cultured in Dulbecco's minimal essential medium (high glucose) supplemented with 10% foetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin, and transfected with rat CRL receptor and human RAMP3 ($pcDNA3_{zeo}$ and $pcDNA3_{G\text{-}418}$ vectors, respectively) as previously described (Choksi et al., 2002).

2.4. Membrane preparation and Western blot analysis

The preparation of cell membranes and their Western blot analysis were performed as previously described (Hagner et al., 2001). The affinity-purified antibody MR003 was used at a dilution of 1:500.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA extraction, reverse transcription and polymerase chain reaction (PCR) were done as previously described (Hagner et al., 2002b).

2.6. Immunohistochemistry of lung and isolated rat endothelial cells

Immunohistochemical analysis was performed on lungs from adult Wistar rats and on the isolated rat lung endothelial cells grown on eight-well chamber slides. Lungs were collected, snap frozen in melting isopentane and stored at −80 °C before being serially sectioned (10 μm) on a cryostat. The subsequent procedures of tissue preparation and immunohistochemical analysis were as previously described (Hagner et al., 2002a). For identification of endothelial cells, a monoclonal antibody to RECA-1 (Serotec, Düsseldorf, Germany) at 1:200 and a monoclonal antibody to platelet endothelial cell adhesion molecule (PECAM) (Pharmingen, Hamburg, Germany) at 1:100 were separately employed. For both antibodies, fluorescent staining was obtained with FITC-coupled donkey anti-mouse IgG (Dianova, Hamburg, Germany) at 1:100.

2.7. Immunohistochemistry of transfected cells

Transfected (CRL receptor + RAMP3) and non-transfected cells were grown in wells on poly-L-lysine-coated glass-slides. Cells were washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (30 min), washed in PBS, then water, before drying and storage at −80 °C. Before immunostaining, slides were thawed to room temperature and then added to PBS containing 0.2% Triton X-100 (30 min), dehydrated using ethanol and treated with 0.3% H_2O_2/100% alcohol (30 min) in order to block endogenous peroxidase activity. Following rehydration in phosphate-buffered saline (PBS), cells were incubated (overnight) with MR003 (1:800) and immunostained using avidin-biotin-immunoperoxidase (ABC) according to the manufacturer's protocol (Novocastra Labs, Newcastle-upon Tyne, UK).

3. Results

A cell line that was stably transfected with cDNAs encoding the rat CRL receptor and human RAMP3 enabled us to characterize the rat CRL receptor antibody MR003 by immunofluorescence and Western blot analysis. For the immunohistochemistry analyses, the specificity of immu-

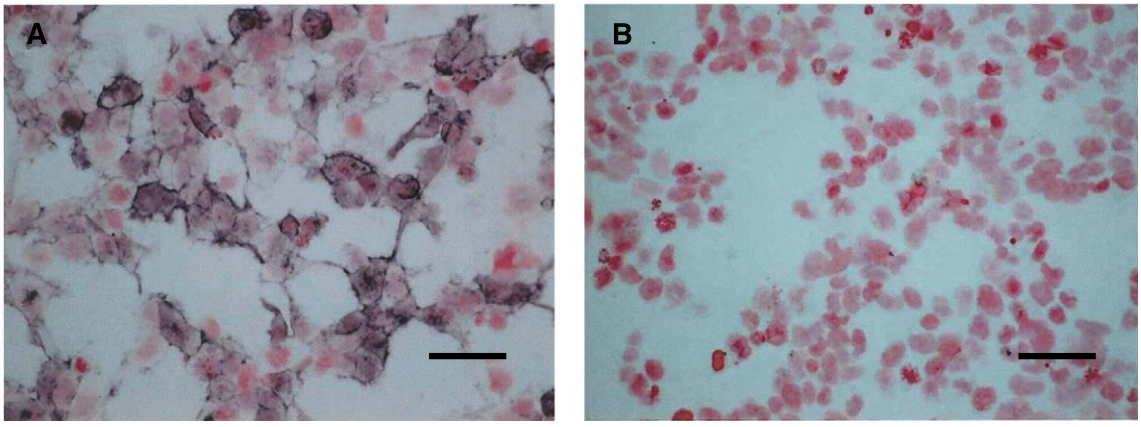

Fig. 1. Immunohistochemical analysis with anti-rat CRL receptor antibody, MR003, of HEK cells co-transfected with rat CRL receptor and human RAMP3 cDNAs (A) (bar = 50 μm). Non-transfected HEK cells exhibit no immunoreactivity (B) (bar = 50 μm).

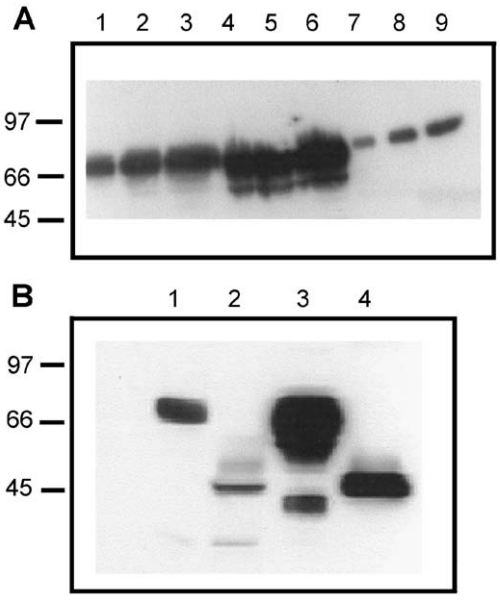

Fig. 2. (A) Western blot analysis with anti-rat-CRL receptor antibody, MR003, of plasma membranes prepared from rat lung (lanes 1, 2, 3, loaded respectively with 5, 10, 20 μl), rat CRL receptor cDNA-transfected HEK cells (lanes 4, 5, 6, loaded respectively with 5, 10, 20 μl), and mouse lung (lanes 7, 8, 9, loaded respectively with 5, 10, 20 μl). (B) Western blot analysis of plasma membranes from rat lung (lanes 1, 2) and from the transfected HEK cells (lanes 3, 4). A shift in the size of the immunoreactive band is revealed following treatment with *N*-glycosidase-F (lanes 2 and 4). Each blot is a representative of three analyses.

nostaining was established by the absence of immunostaining following pre-incubating the antibody with the peptide antigen (data not shown). Fig. 1A shows that the transfected HEK cells express CRL receptor-immunoreactivity, most of which appears associated with the cell membrane, in contrast to non-transfected cells that are negative (Fig. 1B). Fig. 2 presents further characterisation of the antibody, MR003, using Western blot analysis of plasma membranes prepared from transfected cells and from rat lung.

The plasma membranes prepared from CRL receptor-transfected HEK cells contain two immunoreactive fractions of differing molecular size, with the predominant one being of similar size (approximately 70 kDa) to the single CRL receptor-immunoreactive fraction found in rat lung membranes. Also shown in Fig. 2A is evidence of recognition by the antibody, MR003, of mouse CRL receptor, as indicated by the band of CRL receptor-immunoreactivity detected in mouse lung membranes. Following treatment with endoglycosidase-F, Western blot analysis reveals a shift to lower molecular size of each of the immunoreactive bands (Fig. 2B).

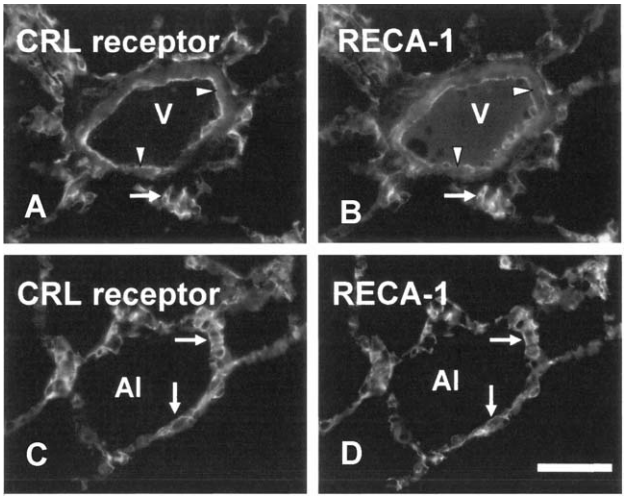

Fig. 3. Fluorescent immunohistochemical analysis of consecutive sections using the antibody MR003 for CRL receptor and an antibody for the endothelial marker protein, RECA-1. Co-stained endothelial cells (arrow heads) of a pulmonary vein (V) and (arrows) of capillaries can be observed (Al = alveolus) (bar = 50 μm).

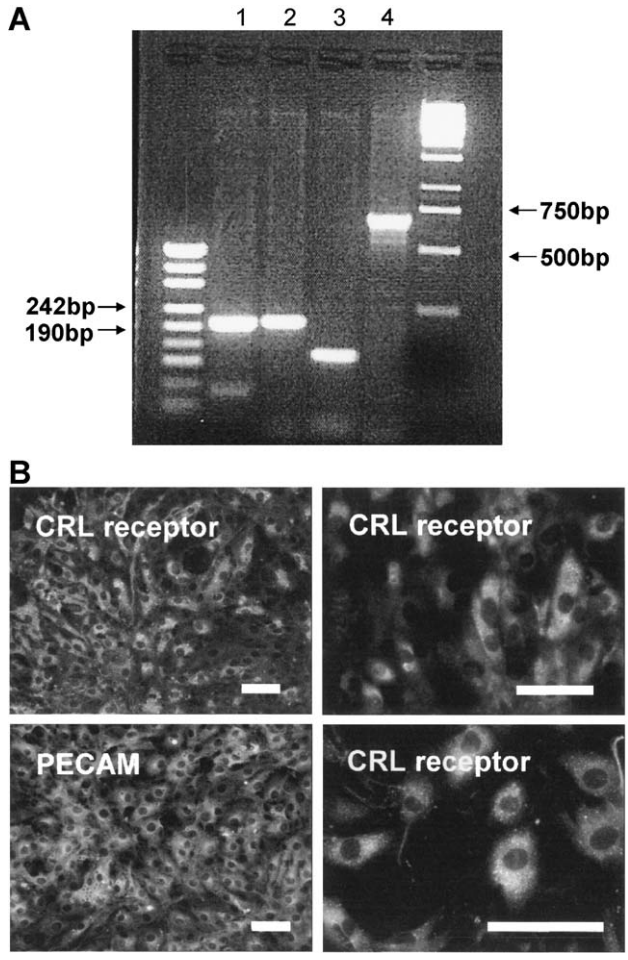

Fig. 4. (A) RT-PCR of isolated rat lung endothelial cells reveals single major bands of the expected size for RAMP1 (197 bp) (lane 1), RAMP2 (203 bp) (lane 2), RAMP3 (120 bp) (lane 3), and CRL receptor (705 bp) (lane 4). The two sets of molecular size marker (pUC19 DNA/*Msp*I/(*Hpa*II) and Gene Ruler 1 kb from MBI GmbH, Germany) were run (in the lanes left and right, respectively, of the test samples). (B) Immunohistochemical analysis with anti-rat CRL receptor antibody, MR003, of isolated rat lung endothelial cells shown in three photomicrographs of different fields at different magnification. The fourth photomicrograph shows immunostaining using an antibody for the endothelial marker protein, PECAM.

In Fig. 3, CRL receptor-specific immunostained endothelial cells are seen in pulmonary vein and in alveolar capillaries in which the immunostaining is particularly intense. A similar distribution of CRL receptor-specific immunostaining was detected throughout the lung. The identity of these CRL receptor-immunoreactive cells as endothelial cells is confirmed by separate staining of these cells with antibodies for the endothelial specific protein marker, RECA-1 (Fig. 3).

Fig. 4A shows the cDNA products obtained from rat microvascular endothelial cells using RT-PCR with gene-specific primers for CRL receptor and for each of the three RAMPs, and a single band of the expected size was detected for each cDNA. The specificity of these bands was established by their absence following omission of the reverse transcriptase or of the Taq polymerase. Virtually all of the isolated rat lung endothelial cells exhibit specific CRL receptor-immunostaining (Fig. 4B) as well as being immuno-positive for the endothelial phenotypic marker, PECAM.

4. Discussion

Here we describe the development and characterisation of an affinity-purified polyclonal antibody for the rat CRL receptor, with which we obtained novel evidence that the rat vascular endothelium expresses the CRL receptor protein as we have shown already in human (Hagner et al., 2002c). Particularly noteworthy is that, similar to our findings in human, the microvascular endothelium appears to express high levels of CRL receptor.

By using immunohistochemistry, we were able to show that our antibody detects rat CRL receptor in HEK cell membranes as is expected following co-transfection of rat CRL receptor cDNA with a RAMP cDNA (McLatchie et al., 1998). RAMPs are required for the incorporation of CRL receptor in to the cell membrane as well as determining ligand specificity. In Western blot analysis, the antibody detects a major and minor immunoreactive band in plasma membranes prepared from these cells. The major band is of similar size to the single CRL receptor-immunoreactive fraction found in rat lung membranes, a tissue known to express high levels of CRL receptor mRNA (Fluhmann et al., 1997). It corresponds to the fully glycosylated CRL receptor-form. The minor band represents a lesser glycosylated form of the receptor, which is in agreement with the size of the described core-glycosylated form (McLatchie et al., 1998). Consistent with this is the shift in size observed following deglycosylation with endoglycosidase-F treatment. The molecular sizes of the detected CRL receptor-immunoreactive bands are also in good agreement with the results of cross-linking experiments (Coppock et al., 1996).

CGRP as well as adrenomedullin (both CRL receptor ligands) exert potent endothelium-dependent vasodilatory action on the pulmonary vasculature (Heaton et al., 1995; Mannan et al., 1995; Nossaman et al., 1995; Han et al., 1997). The endothelium-dependent vasodilatory action of CGRP is a putative protective mechanism against pulmonary hypertension (Keith, 2000; Qing et al., 2001). We could demonstrate that CRL receptor protein is expressed in the rat lung vascular endothelium. We expect this also for other rat vascular beds as we showed in human (Hagner et al., 2002a,c).

Furthermore, we provide novel and definitive evidence that the rat microvasculature expresses the CRL receptor protein. Initially, we confirmed expression of CRL receptor mRNA in isolated microvascular endothelial cells as well as obtaining novel evidence of expression of the mRNAs of all three RAMPs, suggesting fully functional CGRP and/or adrenomedullin receptors (McLatchie et al., 1998). Also, immunochemical analysis provided evidence of the CRL receptor protein in isolated rat lung endothelial cells. The cytoplasm of these cells seems to contain most of the immunostaining. This contrasts with the predominant immunostaining of the cell membrane of the same cells in tissue sections and of the transfected cells. This may be due to the culture conditions preventing normal membrane expression of CRL receptor in isolated endothelial cells. The ability of our antibody to detect membrane expression of CRL receptor probably depends on the amount present in the membrane which, in turn, is dependent on the level of co-expression of RAMPs.

Our findings are an important basis for further experimental investigations in the rat of the role of CRL receptor in vascular functions. The antibody that we describe here will be a valuable tool in such investigations. The presence of CRL receptor in all regions of the microvasculature (Hagner et al., 2002c) suggests that it is involved in various aspects of microvascular function, including angiogenesis (Nikitenko et al., 2000), and in effects that have still to be defined.

References

Born, W., Fischer, J.A., Muff, R., 2002. Receptors for calcitonin gene-related peptide, adrenomedullin, and amylin: the contributions of novel receptor-activity-modifying proteins. Recept. Channels 8, 201–209.

Chang, C.P., Pearse II, R.V., O'Connell, S., Rosenfeld, M.G., 1993. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron 11, 1187–1195.

Choksi, T., Hay, D.L., Legon, S., Poyner, D.R., Hagner, S., Bloom, S.R., Smith, D.M., 2002. Comparison of the expression of calcitonin receptor-like receptor (CRL receptor) and receptor activity modifying proteins (RAMPs) with CGRP and adrenomedullin binding in cell lines. Br. J. Pharmacol. 136, 784–792.

Chu, D.Q., Smith, D.M., Brain, S.D., 2001. Studies of the microvascular effects of adrenomedullin and related peptides. Peptides 22, 1881–1886.

Coppock, H.A., Owji, A.A., Bloom, S.R., Smith, D.M., 1996. A rat skeletal muscle cell line (L6) expresses specific adrenomedullin binding sites but activates adenylate cyclase via calcitonin gene-related peptide receptors. Biochem. J. 318, 241–245.

Duijvestijn, A.M., van Goor, H., Klatter, F., Majoor, G.D., van Bussel, E., van Breda Vriesman, P.J., 1992. Antibodies defining rat endothelial

cells: RECA-1, a pan-endothelial cell-specific monoclonal antibody. Lab. Invest. 66, 459–466.

Fluhmann, B., Lauber, M., Lichtensteiger, W., Fischer, J.A., Born, W., 1997. Tissue-specific mRNA expression of a calcitonin receptor-like receptor during fetal and postnatal development. Brain Res. 774, 184–192.

Hagner, S., Haberberger, R., Kummer, W., Springer, J., Fischer, A., Bohm, S., Goke, B., McGregor, G.P., 2001. Immunohistochemical detection of calcitonin gene-related peptide receptor (CGRPR)-1 in the endothelium of human coronary artery and bronchial blood vessels. Neuropeptides 35, 58–64.

Hagner, S., Haberberger, R.V., Overkamp, D., Hoffmann, R., Voigt, K.H., McGregor, G.P., 2002a. Expression and distribution of calcitonin receptor-like receptor in human hairy skin. Peptides 23, 109–116.

Hagner, S., Knauer, J., Haberberger, R., Goke, B., Voigt, K., McGregor, G.P., 2002b. Calcitonin receptor-like receptor is expressed on gastrointestinal immune cells. Digestion 66, 197–203.

Hagner, S., Stahl, U., Knoblauch, B., McGregor, G.P., Lang, R.E., 2002c. Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. Cell Tissue Res. 310, 41–50.

Han, Z.Q., Coppock, H.A., Smith, D.M., Van Noorden, S., Makgoba, M.W., Nicholl, C.G., Legon, S., 1997. The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium. J. Mol. Endocrinol. 18, 267–272.

Heaton, J., Lin, B., Chang, J.K., Steinberg, S., Hyman, A., Lippton, H., 1995. Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. Am. J. Physiol. 268, H2211–H2215.

Keith, I.M., 2000. The role of endogenous lung neuropeptides in regulation of the pulmonary circulation. Physiol. Res. 49, 519–537.

Mannan, M.M., Springall, D.R., Enard, C., Moradoghli-Haftvani, A., Eddahibi, S., Adnot, S., Polak, J.M., 1995. Decreased endothelium-dependent pulmonary vasodilator effect of calcitonin gene-related peptide in hypoxic rats contrasts with increased binding sites. Eur. Respir. J. 8, 2029–2037.

McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G., Foord, S.M., 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393, 333–339.

Nikitenko, L.L., MacKenzie, I.Z., Rees, M.C., Bicknell, R., 2000. Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. Mol. Hum. Reprod. 6, 811–819.

Nossaman, B.D., Feng, C.J., Cheng, D.Y., Dewitt, B.J., Coy, D.H., Murphy, W.A., Kadowitz, P.J., 1995. Comparative effects of adrenomedullin, an adrenomedullin analog, and CGRP in the pulmonary vascular bed of the cat and rat. Life Sci. 56, L63–L66.

Poyner, D.R., Sexton, P.M., Marshall, I., Smith, D.M., Quirion, R., Born, W., Muff, R., Fischer, J.A., Foord, S.M., 2002. International Union of Pharmacology: XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol. Rev. 54, 233–246.

Qing, X., Svaren, J., Keith, I.M., 2001. mRNA expression of novel CGRP1 receptors and their activity-modifying proteins in hypoxic rat lung. Am. J. Physiol., Lung Cell. Mol. Physiol. 280, L547–L554.