Polarity and lipid raft association of the components of the ciliary neurotrophic factor receptor complex in Madin-Darby canine kidney cells

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Summary

Ciliary neurotrophic factor (CNTF) signals via a tripartite receptor complex consisting of the glycosyl-phosphatidylinositol (GPI)-anchored CNTF receptor (CNTF-R), the leukaemia inhibitory factor receptor (LIF-R) and the interleukin-6 (IL-6) signal transducer gp130. We have recently reported that gp130 is endogenously expressed in the polarised epithelial model cell line Madin-Darby canine kidney (MDCK) and we have demonstrated a preferential basolateral localisation of this protein. In the present study we show that MDCK cells also express the LIF-R and respond to stimulation with human LIF by activation of tyrosine phosphorylation of signal transducer and activator of transcription-3 (STAT3), both however in an unpolarised fashion. This suggests that MDCK cells may be target cells for LIF. We have furthermore stably expressed the human CNTF-R in MDCK cells and by two different assays we found an apical localisation. Consistent with these findings, stimulation of CNTF-R-positive cells resulted only in an activation of STAT3 when CNTF was added apically. These data demonstrate that each subunit of the CNTF receptor complex has a distinct distribution in polarised cells which may reflect the different roles the respective cytokines play in vivo.

Since it is currently believed that lipid rafts are involved in signal transduction as well as protein sorting we studied the association of the three receptor complex components with membrane rafts using different protocols. Whereas the CNTF-R cofractionated quantitatively with lipid rafts independently of the method used, gp130 and the LIF-R were found to associate with lipid rafts only partially when detergents were used for isolation. These findings could indicate that either the three receptor complex subunits are localised to the same kind of raft but with different affinities to the liquid-ordered environment, or that they are localised to different types of rafts. CNTF-, LIF-, and IL-6-dependent STAT3 activation was sensitive to the cholesterol-depleting drug methyl-β-cyclodextrin (MCD) suggesting that the integrity of lipid rafts is important for IL-6-type cytokine-induced STAT activation.

Key words: Lipid rafts, Signal transduction, Cell polarity, Cytokines, Interleukin-6

Introduction

Ciliary neurotrophic factor (CNTF) belongs to the family of interleukin-6 (IL-6)-type cytokines and is only released by injured neuronal cells (Heinrich et al., 1998; Sendtner et al., 1994). The specific CNTF receptor (CNTF-R) is a glycosyl-phosphatidylinositol (GPI)-anchored protein (Davis et al., 1991) which is mainly expressed in neuronal cells. The CNTF-induced signal transduction promotes survival and/or differentiation of these cells (Sendtner et al., 1994). Additionally, CNTF can induce synthesis of acute-phase proteins (Heinrich et al., 1998) and also acts on muscle cells (Helgren et al., 1994).

The functional tripartite membrane CNTF receptor complex comprises the ligand-binding CNTF-R and two transmembraneous signalling compounds, the leukaemia inhibitory factor (LIF) receptor and the common subunit for the IL-6-type cytokines gp130 (Stahl and Yancopoulos, 1994). The related cytokine LIF directly binds to the LIF receptor and also recruits gp130 (Gearing et al., 1992). Recently, a novel IL-6-type cytokine, novel neurotrophin-1/B cell stimulating factor-3 (Senaldi et al., 1999) or cardiotrophin-like cytokine was found to interact with a soluble receptor, the cytokine-like factor-1 (Elson et al., 2000), forming a heteromeric ligand for the functional CNTF receptor complex (Plun-Favreau et al., 2001). In any case, ligand-induced heterodimerisation of gp130 and LIF-R results in the activation of associated Janus kinases (JAK) that phosphorylate the signal transducing receptor chains thereby creating docking sites for transcription factors of the STAT (signal transducer and activator of transcription) family, STAT3 and STAT1. STAT proteins in turn become tyrosine phosphorylated, dimerise, and translocate to the nucleus where they bind to specific response elements of CNTF target genes (Heinrich et al., 1998).

Like many other differentiated cells neurons are polarised cells, i.e. they have two different plasma membranes: the somato-dendritic membrane, which is responsible for the reception of signals and which includes many neurotransmitter and ion-channel receptors, and the axonal membrane domain,

which contains GPI-anchored cell-adhesion molecules (Rodriguez-Boulan and Powell, 1992) and receptors for neurotransmitters (Pan, 2001) and hormones (Bishop et al., 2000). In simple epithelial cells two plasma membrane domains with a distinct protein and lipid composition are separated by tight junctions: the apical membrane acting as a barrier towards the external milieu (lumen) and the basolateral membrane facing neighbouring cells and the extracellular matrix (Eaton and Simons, 1995). Dotti and Simons suggested that in neuronal cells the sorting of axonal and somato-dendritic proteins to their respective membrane domain is achieved by similar mechanisms as the sorting of apical and basolateral proteins in epithelial cells (Dotti et al., 1991; Dotti and Simons, 1990). Thus, they found that viral glycoproteins that are expressed at either the apical or basolateral domain of polarised Madin-Darby canine kidney (MDCK) cells are targeted to the axonal or somato-dendritic membrane of hippocampal neurons, respectively. GPI-anchored proteins that are sorted apically in MDCK cells were found to be exclusively axonal. Since it is difficult to analyse sorting in neurons directly, MDCK cells have served in the past as a reasonable if limited model (Jareb and Banker, 1998).

GPI-anchors in combination with lipid rafts have been discussed as being responsible for targeting proteins to the apical membrane in polarised cells (Weimbs et al., 1997). These microdomains contain a special subset of lipids, e.g. sphingolipids and cholesterol to which some types of membrane proteins associate, especially lipid-modified ones such as GPI-anchored or acylated proteins (Simons and Ikonen, 1997). Lipid rafts accumulate especially at the apical surface of polarised cells and in the axonal membrane domain of neurons, accordingly (Simons and Ikonen, 1997). However, basolateral membranes also contain lipid rafts but in smaller amounts. Basolateral membranes contain specialised invaginated lipid rafts termed caveolae whereas the apical surface of epithelial cells does not. Lipid rafts are not only relevant in intracellular transport but also in signalling events at the plasma membrane (Simons and Toomre, 2000).

We have recently analysed the expression of both components of the human interleukin-6 receptor complex gp80 and gp130 in polarised MDCK cells and found that both subunits are preferentially expressed at the basolateral membrane (Martens et al., 2000). The CNTF-R is the only GPI-linked protein within the IL-6-type receptor family. This feature suggests that the CNTF-R may be sorted differently than gp130, namely apically in MDCK cells and probably axonally in neurons. In addition the localisation of the LIF-R has to be determined. To this end, we have generated MDCK cells stably expressing the human CNTF-R. We have compared its polarised expression with that of endogenous gp130 and LIF-R and studied whether the three components associate with detergent-resistant lipid rafts. Our results indicate that each of the three receptor components has a distinct distribution that may reflect the different roles the respective cytokines IL-6, CNTF and LIF play in processes such as inflammation, cell survival, development of cell polarity and implantation.

Materials and Methods

Materials

All chemicals were supplied by Sigma (Taufkirchen, Germany) unless otherwise indicated.

Restriction enzymes and T4 ligase were obtained from Boehringer (Mannheim, Germany), prestained protein molecular mass markers and a prestained protein ladder were from MBI Fermentas (St. Leon-Rot, Germany) and lovastatin was from VWR International (Darmstadt, Germany). Normal donkey and goat serum were supplied by Jackson ImmunoResearch (West Grove, PA, USA), and normal rabbit serum by DAKO (Hamburg, Germany). Protein A Sepharose™ CL-4B, Full-Range Rainbow™ recombinant molecular mass markers and the ECL+plus western blotting detection system were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Protein G Sepharose, protease inhibitor cocktail, cholesterol, cholesterol oxidase from *Streptomyces* sp. and horseradish peroxidase were obtained from Sigma (Taufkirchen, Germany), and aprotinin, leupeptin and pepstatin A from Serva (Heidelberg, Germany). EZ-Link™ sulfo-N-hydroxysuccinimidyl-biotin (sulfo-NHS-biotin) and the streptavidin-horseradish peroxidase (HRP) conjugate were obtained from Pierce (Rockford, IL, USA). Pansorbin® cells were supplied by Merck (Darmstadt, Germany) and the Slow Fade® Light Antifade Kit by MoBiTec (Göttingen, Germany). Dulbecco's modified Eagle's medium (DMEM), DMEM/HAM's F12, phosphate-buffered saline (PBS), bovine serum albumin (BSA) and G418 sulphate were purchased from PAA (Cölbe, Germany). Foetal calf serum (FCS), penicillin/streptomycin and trypsin/EDTA were obtained from Biochrom (Berlin, Germany). Recombinant IL-6 and soluble IL-6R (sIL-6R) were prepared as described previously (Weiergräber et al., 1995). CNTF was supplied by Biocarta (Hamburg, Germany), LIF by Sigma (Taufkirchen, Germany). Antibodies were obtained as follows: mouse α-caveolin-1 from Transduction Laboratories (San Jose, CA, USA), rabbit α-caveolin-1 (N-20), mouse α-CNTF-R (AN-B2) and rabbit α-LIF-R (C-19) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), mouse α-gp130 (clone B-P4) from Diaclone (Besançon, France), HRP-linked anti-rabbit IgG and HRP-linked anti-mouse IgG from Amersham Pharmacia Biotech (Freiburg, Germany), rabbit α-pY-STAT3 (Tyr 705) from New England Biolabs (Frankfurt/Main, Germany), and rabbit α-Rab5 from StressGen Biotechnologies (Victoria, BC, Canada). Mouse α-LIF-R (7G7) antibody was kindly provided by V. Pitard (Bordeaux, France). All other antibodies were purchased from DAKO (Hamburg, Germany). pCB6 cDNA and the cDNA for human CNTF-R were kindly provided by A. Le Bivic (Marseille, France) and J. Weis (Berne, Switzerland) respectively.

Expression vectors

The cDNA of the human CNTF-R was subcloned as a *Xba*I/*Bam*HI fragment from the pSVL vector into the expression vector pCB6 (Brewer and Roth, 1991) where it is under the control of the cytomegalovirus immediate-early promoter.

Culture and transfection of cell lines

MDCK cells, strain II, were grown in DMEM supplemented with 10% FCS, streptomycin (100 mg/l) and penicillin (60 mg/l) at 37°C in a 5% CO_2 water-saturated atmosphere. For polarity studies, cells were plated and grown for 5 days on Costar Transwell filters (van Meer et al., 1987). Transfection of MDCK cells was performed by a modification of the calcium-phosphate precipitation procedure as previously described (Graham and van der Eb, 1973; Rodriguez-Boulan et al., 1989). The transfected cells were selected with 500 μg/ml G418 for 10-14 days. The populations were screened for expression by indirect immunofluorescence staining. Transfected cells were subcloned using limiting dilution. Two positive clones were selected for further experiments.

HeLa cells were cultured in DMEM supplemented with 10% FCS and streptomycin/penicillin. HepG2 cells were cultured in DMEM/HAM's F12 containing 10% FCS and penicillin/streptomycin. Ba/F3-gp130AA (LIF-R-negative control cells) and

Ba/F3-LIF-R cells were grown in DMEM containing 10% FCS, penicillin/streptomycin, and 5% conditioned medium from X63AG-653 BPV-mIL-3 myeloma cells as a source of IL-3 (Thiel et al., 1999).

Cytokine stimulation and treatment with methyl-β-cyclodextrin (MCD) of MDCK cells

MDCK cells were grown for 5 days on Transwell filters or for 3-5 days on 100 mm dishes and starved of serum 16 hours before the respective experiment. After two washes with PBS the cells were stimulated (apically or basolaterally when grown on Transwell filters) with IL-6 (20 ng/ml) and sIL-6R (0.5 μg/ml), LIF (50 ng/ml), or CNTF (25 ng/ml) in DMEM supplemented with 0.2% BSA for 15-30 minutes at 37°C.

For cholesterol depletion cells were preincubated with lovastatin (4 μM) and mevalonate (0.25 mM). Control cells not receiving MCD treatment were not preincubated with lovastatin and mevalonate. After 48 hours the cells were treated with 10-20 mM MCD in DMEM with 0.2% BSA and lovastatin (4 μM) at 37°C for different times as indicated in the figures.

Determination of cholesterol in cell lysates

MDCK cells treated as described above were lysed for 30 minutes at room temperature in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, PMSF (100 μg/ml) and aprotinin (5 μg/ml). The cells were homogenised using a 20G-syringe and 2 ml aliquots were subjected to lipid extraction following the method of Bligh and Dyer (Bligh and Dyer, 1959). Briefly, lipids were extracted by adding chloroform/methanol 2:1 (v/v). After mixing vigorously and centrifuging for 5 minutes at 1000 ***g*** the lower phase was collected and washed twice with water/methanol 1:1 (v/v). Aliquots of the washed chloroform phases were dried under N_2 and dissolved in 50 μl ethanol. To measure cholesterol, 1 ml 0.1 M phosphate buffer, pH 7.2, containing 0.3 U/ml cholesterol oxidase, 1 U/ml peroxidase, 0.3 μmol/ml amino-4-antipyrine, 30 μmol/ml hydroxybenzene sulphonate, 0.5% Triton X-100, and 20 mM cholic acid, was added to each sample, incubated for 5 minutes at 37°C, and the absorption was measured at 500 nm.

Immunofluorescence staining

Transfected MDCK-CNTF-R cells were grown for 5 days on collagen-coated coverslips (~10 μg/cm^2 of a 1% collagen solution) and fixed with 2% (w/v) paraformaldehyde as described previously (Graeve et al., 1990). After permeabilisation with 0.1% Triton X-100 and blocking with PBS^{++} (PBS with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) containing 1% BSA and a 1/10 dilution of normal serum from goat and rabbit, the cells were incubated with a CNTF-R-specific mouse antiserum and simultaneously with a caveolin-1-specific rabbit antiserum. Bound antibodies were detected using a Cy3-conjugated anti-mouse IgG antibody (CNTF-R) and a FITC-conjugated anti-rabbit IgG antibody (caveolin-1). Coverslips were mounted on slides using the Slow Fade® Light Antifade Kit. For negative controls, samples were incubated with the combination of secondary antibodies alone. Microscopy was performed using the Zeiss Axiovert 100M confocal laser scanning system equipped with LSM 5 Pascal, a helium-neon laser, an argon laser, and a Plan Apochromat 100× objective (Carl Zeiss, Jena, Germany). Specific emission filters were used for each fluorescent wavelength (FITC: 505-530 nm, Cy3: 560-615 nm).

Biotinylation assay

Sulfo-NHS-biotin was employed to label cell surface proteins (Graeve et al., 1989). MDCK cells were grown on Transwell filters for 5 days, then washed three times with PBS^{++} and once with biotinylation buffer (120 mM NaCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 8.5) at 4°C for 15 minutes. Sulfo-NHS-biotin labelling (0.5 mg/ml in biotinylation buffer) was performed twice for 20 minutes at 4°C either basolaterally or apically, with PBS^{++} on the opposite site. Cells were washed with DMEM supplemented with 0.2% BSA and 20 mM Hepes, pH 7.4, and three times with PBS^{++} for 5 minutes at 4°C each.

Immunoprecipitation

Cells were solubilised in 1 ml lysis buffer (1% Nonidet P40, 10 mM Tris-HCl, pH 7.4, 60 mM EDTA, 0.4% deoxycholic acid) in the presence of protease inhibitors (0.75 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) for 30 minutes at 4°C. Insoluble material was removed by centrifugation and the supernatants were pretreated with Pansorbin® cells for 1 hour at 4°C. SDS was added to a final concentration of 0.3%. After incubation with either a CNTF-R-specific mouse antiserum (IgG_{2a}), a LIF-R-specific rabbit antiserum, or a LIF-R-specific mouse antiserum for at least 2 hours at 4°C the immune complexes were precipitated with protein A Sepharose or protein G Sepharose (5 mg/ml in washing buffer: 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). After centrifugation the Sepharose beads were washed four times with washing buffer and boiled in reducing sample buffer. The eluted proteins were separated on an 8% SDS-polyacrylamide gel.

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously (Andrews and Faller, 1991). The protein concentration was determined by a Bio-Rad® protein assay. To investigate the activation of STAT3 identical protein aliquots were analysed by 10% SDS-PAGE and western blotting. Bands were densitometrically analysed using the EASY Win 32 gel documentation system from Herolab (Wiesloch, Germany).

Detergent-free purification of caveolin-1 rich membrane fractions

MDCK cells grown to confluency in 100 mm dishes were used to prepare caveolin-1-enriched membrane fractions (Song et al., 1996). After two washes with ice-cold PBS the cells were scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenisation was carried out subsequently (loose fitting Dounce homogeniser, 10 strokes; Polytron tissue grinder, three 10-second bursts; sonicator, three 20-second bursts). The homogenate was then adjusted to 45% (w/v) sucrose by addition of 2 ml 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and a 5-35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose and 4 ml of 35% sucrose, both in MBS containing 250 mM sodium carbonate). After ultracentrifugation (4°C, 192,000 ***g*** for 16-20 hours in a SW40 rotor; Beckman Instruments, USA) 1 ml fractions were collected and precipitated with trichloroacetic acid (TCA). The proteins were separated by SDS-PAGE (10% acrylamide) and transferred to a poly-vinylidene difluoride (PVDF) membrane.

Detergent extraction of MDCK cells using Triton X-100 or Brij 58

After two washes with ice-cold PBS the cells were lysed for 20 minutes in 2 ml TNE buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA) containing a protease inhibitor cocktail and 1% Triton X-100 or 1% Brij 58, respectively (Brown and Rose, 1992; Roepstorff et al., 2002). The cells were scraped with a rubber policeman, homogenised using 10 strokes of a Dounce homogeniser and sonicated by three 10-second bursts. The lysate was adjusted to 40%

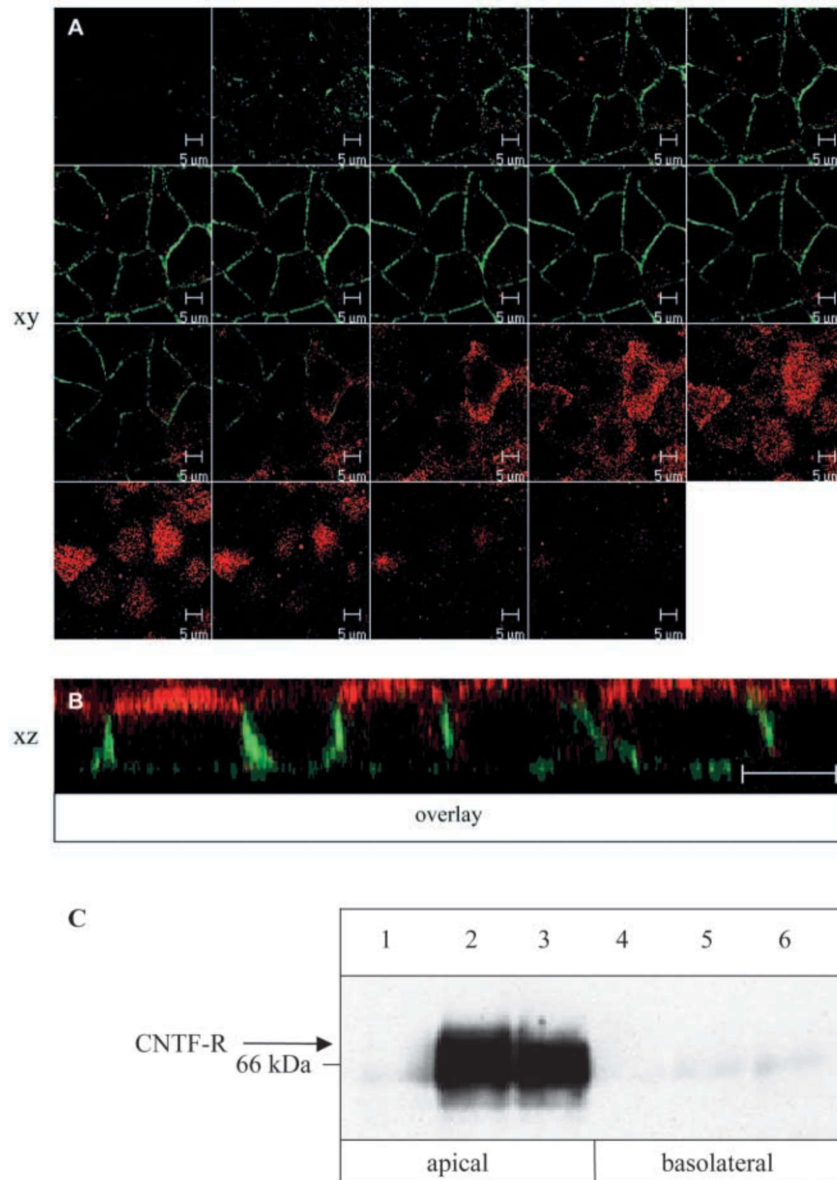

Fig. 1. Localisation of the human CNTF-R and endogenous caveolin-1 in stably transfected MDCK cells. (A,B) MDCK cells stably transfected with the human CNTF-R were grown on collagen-coated coverslips for 5 days. Indirect immunofluorescence staining was performed using specific antibodies against the CNTF-R and caveolin-1 followed by Cy3-conjugated (CNTF-R) and FITC-conjugated (caveolin-1) secondary antibodies. Microscopy was performed using a Zeiss Axiovert 100 M confocal laser scanning microscope equipped with LSM 5 Pascal (Jena, Germany). (A) The xy-scan shows the localisation of the human CNTF-R (red) and caveolin-1 (green) in serial sections of 0.4 µm from the basal to the apical pole. (B) The xz-scan for CNTF-R and caveolin-1. (C) Parental MDCK (lanes 1 and 4) and stably transfected MDCK-CNTF-R cells (lanes 2+3 and 5+6) were grown on Transwell filters for 5 days. Sulfo-NHS-biotin was employed to selectively label the apical or the basolateral surface. The cells were extracted with lysis buffer and the supernatants were immunoprecipitated with a monoclonal CNTF-R-specific antibody. Immunoprecipitates were analysed by SDS-PAGE and western blot. A molecular mass marker containing albumin (66 kDa) was used for comparison. The biotinylated proteins were detected using HRP-conjugated streptavidin and visualised by ECL+plus. For MDCK-CNTF-R cells two independent determinations are shown.

sucrose by addition of 2 ml 80% sucrose prepared in TNE with protease inhibitors and a discontinuous sucrose gradient was formed above (4 ml 30% sucrose and 4 ml 5% sucrose, both in TNE containing protease inhibitors but without detergents). Gradients were centrifuged and processed as described above.

Western blotting and immunodetection

Electrophoretically separated proteins were transferred to PVDF membranes by the semi-dry western blotting method. When detecting biotinylated proteins non-specific binding was blocked with 5% BSA in TBS-N (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Nonidet P-40) for 1 hour. Otherwise 5% skimmed milk powder in TBS-T (0.1% Tween 20) was used. The blots were subsequently incubated with a CNTF-R-specific mAb, a gp130-specific mAb (clone B-P4) or a LIF-R-specific pAb, respectively, and with a caveolin-1-specific mAb or a Rab5-specific pAb followed by incubation with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies, each for 1 hour. To detect biotinylated CNTF-R or LIF-R the immunoblot was incubated with HRP-conjugated streptavidin in TBS-N for 1 hour. When detecting activated STAT3, membranes were probed with an activation-specific antibody against STAT3 followed by HRP-conjugated goat anti-rabbit IgG antibody. The immunoblots were developed using the enhanced chemiluminescence system (ECL+plus) according to the manufacturer's instructions.

Results

The CNTF receptor is localised to the apical plasma membrane of MDCK cells

Since the ciliary neurotrophic factor (CNTF) receptor is mainly expressed in neurons with a polar morphology we were interested in the sorting of this cytokine receptor in polarised cells. For this study we used the polarised cell line Madin-Darby canine kidney (MDCK), which is a well-established model for the analysis of sorting and targeting of plasma membrane proteins (Rodriguez-Boulan and Powell, 1992). Endogenous CNTF-R was not detectable in these cells either by immunofluorescence or by a functional assay (data not shown). MDCK cells were stably transfected with a cDNA coding for the human wild-type CNTF receptor (MDCK-CNTF-R) as outlined in the Materials and Methods section. In order to assess the polarity of expression, cells were grown on collagen-coated coverslips for 5 days, fixed, permeabilised and processed for indirect immunofluorescence staining using a monoclonal antibody against the human CNTF-R followed by incubation with a Cy3-conjugated secondary antibody. The cells were costained using a polyclonal antibody against caveolin-1 and a FITC-conjugated secondary antibody. Stained cells were analysed by confocal laser scanning microscopy (Fig. 1A,B). Analysis of

serial sections obtained by xy-scanning showed a basal and typical lateral rim-like staining for caveolin-1 (Fig. 1A, green fluorescence). Surprisingly, in most cells no apical staining of caveolin-1 was detected which is in contrast to published data (Scheiffele et al., 1998). Different expression levels of endogenous caveolin-1 in different MDCK clones most probably account for this difference since analysis of MDCK cells from another laboratory showed apical staining for caveolin-1 in most cells (data not shown). In contrast to caveolin-1, staining for the CNTF-R was even at the apical membrane (Fig. 1A, red fluorescence). The overlays did not show any colocalisation of the two proteins (Fig. 1A). This can also clearly be seen in the xz-scan where no overlap between the apical CNTF-R and the basolateral caveolin-1 occurs (Fig. 1B). From these results we conclude that the CNTF-R is mainly localised to the apical domain of MDCK cells.

In order to corroborate this finding we used a biochemical approach. Stable transfectants and parental cells were cultured on Transwell filters for 5 days prior to the experiment. The polarity of the CNTF-R was assessed by selective surface-labelling of either the apical or basolateral plasma membrane with sulfo-NHS-biotin (Graeve et al., 1989; Zurzolo et al., 1993). The CNTF-R was immunoprecipitated, analysed by SDS-PAGE and transferred to a PVDF membrane. Biotinylation of the CNTF-R was detected using HRP-conjugated streptavidin for an enhanced chemiluminescence reaction (Fig. 1C). Labelling of the apical side resulted in a prominent CNTF-R signal (Fig. 1C, lanes 2 and 3). The recognised protein migrates at an apparent molecular mass a little above 66 kDa (albumin), which is in accordance with the observed size of the human CNTF-R [72 kDa (Davis et al., 1991)]. Labelling of the basolateral plasma membrane only resulted in a very weak signal (Fig. 1C, lanes 5 and 6) comparable to the background signal in the parental control MDCK cells (Fig. 1C, lanes 1 and 4).

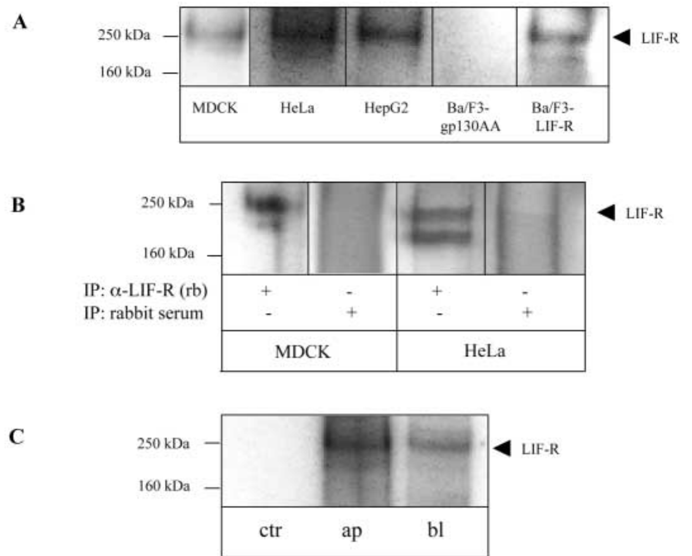

Fig. 2. (A) Non-polarised expression of LIF-R in MDCK cells. HeLa, HepG2, Ba/F3-gp130AA, and Ba/F3-LIF-R cells or MDCK cells were extracted with lysis buffer and cell lysates immunoprecipitated with a monoclonal LIF-R specific antibody (7G7) or a LIF-R-specific rabbit antiserum, respectively. (B) MDCK and HeLa cells were grown on tissue culture dishes, lysed and incubated either with a LIF-R-specific rabbit antiserum [α-LIF-R (rb) +] or non-immune rabbit serum (rabbit serum +). (C) MDCK cells were grown on Transwell filters for 5 days. Sulfo-NHS-biotin was employed to selectively label the apical (ap) or the basolateral (bl) surfaces or cells were left untreated (ctr). The lysates were incubated with a LIF-R-specific rabbit antiserum. Immunoprecipitates (A-C) were analysed by SDS-PAGE and western blotting using either a polyclonal LIF-R-specific antibody followed by an appropriate HRP-conjugated secondary antibody (A,B) or HRP-conjugated streptavidin (C). Blots were developed using ECL+plus.

The LIF-R is expressed non-polarised in MDCK cells

A functional CNTF receptor complex consists of the CNTF-R as a ligand binding subunit and a heterodimer of gp130 and the LIF-R as signal transducer. From previous experiments it was clear that MDCK cells express gp130 endogenously and that canine gp130 is able to interact with human IL-6/sIL-6 receptor complexes resulting in activation of the Jak/STAT pathway (Martens et al., 2000). However, detection of canine gp130 by immunofluorescence staining or by the biotinylation assay described above was not possible because of the lack of specific antibodies. Therefore, we over-expressed human gp130 in MDCK cells and found a preferential basolateral localisation (Martens et al., 2000). The presence of a functional LIF-R in MDCK cells has not been analysed so far. We tested whether antibodies against the human LIF-R would detect the canine LIF-R. When used on human cells (HeLa, HepG2) or Ba/F3 cells transfected with the LIF-R two different antibodies specifically detected a protein migrating with a molecular mass of between 200 and 250 kDa (Fig. 2A) which is in good accordance with the published size of glycosylated LIF-R (Gearing et al., 1991). A protein of identical size could be detected in MDCK cells (Fig. 2A, left lane). To further verify the identity of the detected protein, MDCK and HeLa cell lysates were immunoprecipitated with a LIF-R-specific rabbit antiserum or a non-immune rabbit serum instead (Fig. 2B). In both MDCK and HeLa cells again a protein migrating between 200 and 250 kDa could be detected in the specific immunoprecipitate (Fig. 2B). To assess the polarity of the endogenous LIF-R in MDCK cells we selectively labelled surface proteins with sulfo-NHS-biotin and analysed the proteins by immunoprecipitation and western blotting as described above. As shown in Fig. 2C, the endogenous LIF-R is unexpectedly localised to both the apical and the basolateral plasma membrane in MDCK cells.

Polarity of STAT3 activation in MDCK cells

Next we tested whether the transfected CNTF-R and the endogenous LIF-R are functional in MDCK cells. Confluent parental MDCK, MDCK-CNTF-R and MDCK-gp130 cells were stimulated apically with CNTF or LIF or left untreated and tyrosine phosphorylation of STAT3 was studied by western blotting of nuclear extracts using an activation-specific STAT3 antibody (Fig. 3). A small but reproducible fraction of activated nuclear STAT3 was already detected in unstimulated cells (Fig. 3, left lane). This basal activity was reproducibly observed even when MDCK cells were starved of serum for 48 hours (data not shown) suggesting that this activation is either a genuine

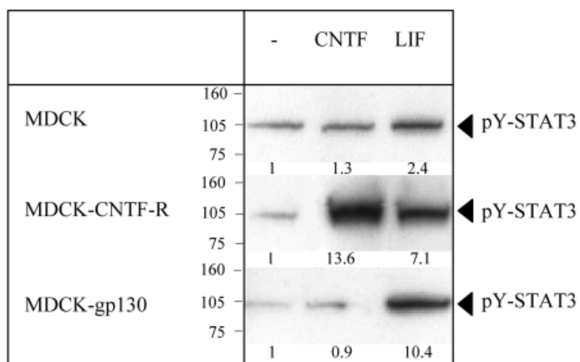

Fig. 3. Functionality of the CNTF-R and the LIF-R in MDCK cells. Parental MDCK (upper panel), stably transfected MDCK-CNTF-R (middle panel) or MDCK-gp130 cells (lower panel) were grown on Transwell filters for 5 days. The cells were apically stimulated with CNTF (25 ng/ml) or LIF (50 ng/ml) for 15-30 minutes at 37°C. Nuclear extracts were prepared and equal protein aliquots analysed by SDS-PAGE and western blotting. Phosphorylated STAT3 proteins were detected with an activation-specific STAT3 antibody (pY-STAT3), an HRP-conjugated secondary antibody and visualised using the ECL+plus system. Quantitation over basal levels (fold stimulation) was calculated in each set of experiment as indicated in the figure.

property of MDCK cells or is due to the production of an autocrine factor. In fact, the latter possibility is supported by the observation that conditioned medium from MDCK cells, when applied to HepG2 cells, caused a prominent STAT3 activation (data not shown). Identification of this factor is difficult because neutralising antibodies to canine cytokines are usually not available.

In parental MDCK cells only stimulation by apically added LIF resulted in a significant increase in STAT3 activation, demonstrating that the endogenous LIF-R is functional and can be activated by human LIF (Fig. 3, MDCK, upper panel). The small increase seen with CNTF was not reproducible (see also Fig. 4). In MDCK-CNTF-R cells both CNTF and LIF caused STAT3 phosphorylation (Fig. 3, MDCK-CNTF-R, middle panel). Thus, also the transfected CNTF-R is functional and can interact with endogenous gp130 and LIF-R. In MDCK overexpressing human gp130 again LIF but not CNTF activated the JAK/STAT pathway (Fig. 3, MDCK-gp130, lower panel). Firstly, these results confirm that the CNTF-R is localised apically in MDCK-CNTF-R cells. Secondly, they imply that endogenous LIF-R can also signal from the apical side of these polarised cells. However, they also raise a paradox: both CNTF and LIF require gp130 for signal transduction, which is mainly targeted basolaterally as was shown recently, and is therefore not available for apically added IL-6/soluble IL-6 receptor complexes (Martens et al., 2000).

To study this in more detail the different MDCK transfectants were stimulated with the different cytokines from either side (Fig. 4). When stimulated with IL-6/sIL-6R all three MDCK cell lines only showed a prominent activation of STAT3 when the stimulus was added basolaterally (Fig. 4, middle panel). In some cases a marginal increase was seen at the apical side, however, this varied from experiment to experiment. As expected, IL-6-induced basolateral stimulation of STAT3 was stronger in MDCK-gp130 cells since more gp130 molecules are available in these cells. With CNTF only MDCK-CNTF-R cells showed an activation of STAT3 and only when stimulated from the apical side (Fig. 4, left panel). In contrast, LIF prominently activated STAT3 in all cell types, both from the apical and basolateral sides (Fig. 4, right panel), as expected from its non-polarised expression shown in Fig. 2C. However, in all cell types stimulation by LIF was stronger from the basolateral side than from the apical side, which is most probably because of the excess of gp130 at the basolateral membrane and the fact that it is limiting on the apical side.

The components of the CNTF receptor complex are differentially localised to lipid rafts

The CNTF-R is linked to the plasma membrane via a GPI-anchor. A role for lipid rafts as transport units for the sorting of GPI-anchored proteins to the apical plasma membrane has been suggested (Brown and Rose, 1992; Muniz and Riezman, 2000; Weimbs et al., 1997). To study whether the CNTF-R in MDCK cells is associated with lipid rafts we initially analysed parental MDCK and MDCK-CNTF-R cells grown to confluency on plastic dishes, using three different established protocols: one employing sodium bicarbonate extraction and two using detergents (Triton X-100 and Brij 58) (Brown and Rose, 1992; Roepstorff et al., 2002). The homogenates were adjusted to either 45% sucrose or 40% sucrose and a discontinuous sucrose gradient was formed above (5-

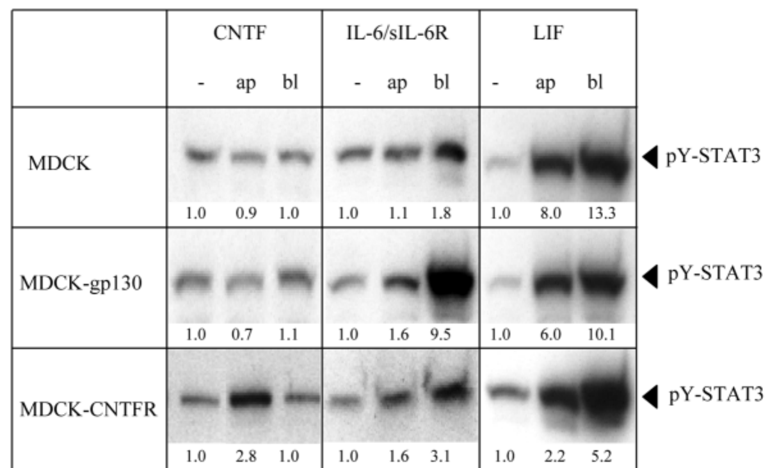

Fig. 4. STAT activation in polarised MDCK cells. Parental MDCK (upper panels) and stably transfected MDCK (MDCK-gp130, middle panels; MDCK-CNTF-R, lower panels) were cultivated on Transwell filters for 5 days. CNTF (25 ng/ml), LIF (50 ng/ml), or IL-6 (20 ng/ml)/ soluble IL-6R (500 ng/ml), respectively, were added to the apical (ap) or the basolateral (bl) surfaces for 30 minutes at 37°C. Nuclear extracts were prepared and analysed by SDS-PAGE and western blotting. Phosphorylated STAT3 proteins were detected with an activation-specific STAT3 antibody (pY-STAT3), an HRP-conjugated secondary antibody and visualised using the ECL+plus system. Quantitation over basal levels (fold stimulation) was calculated in each set of experiment as indicated in the figure.

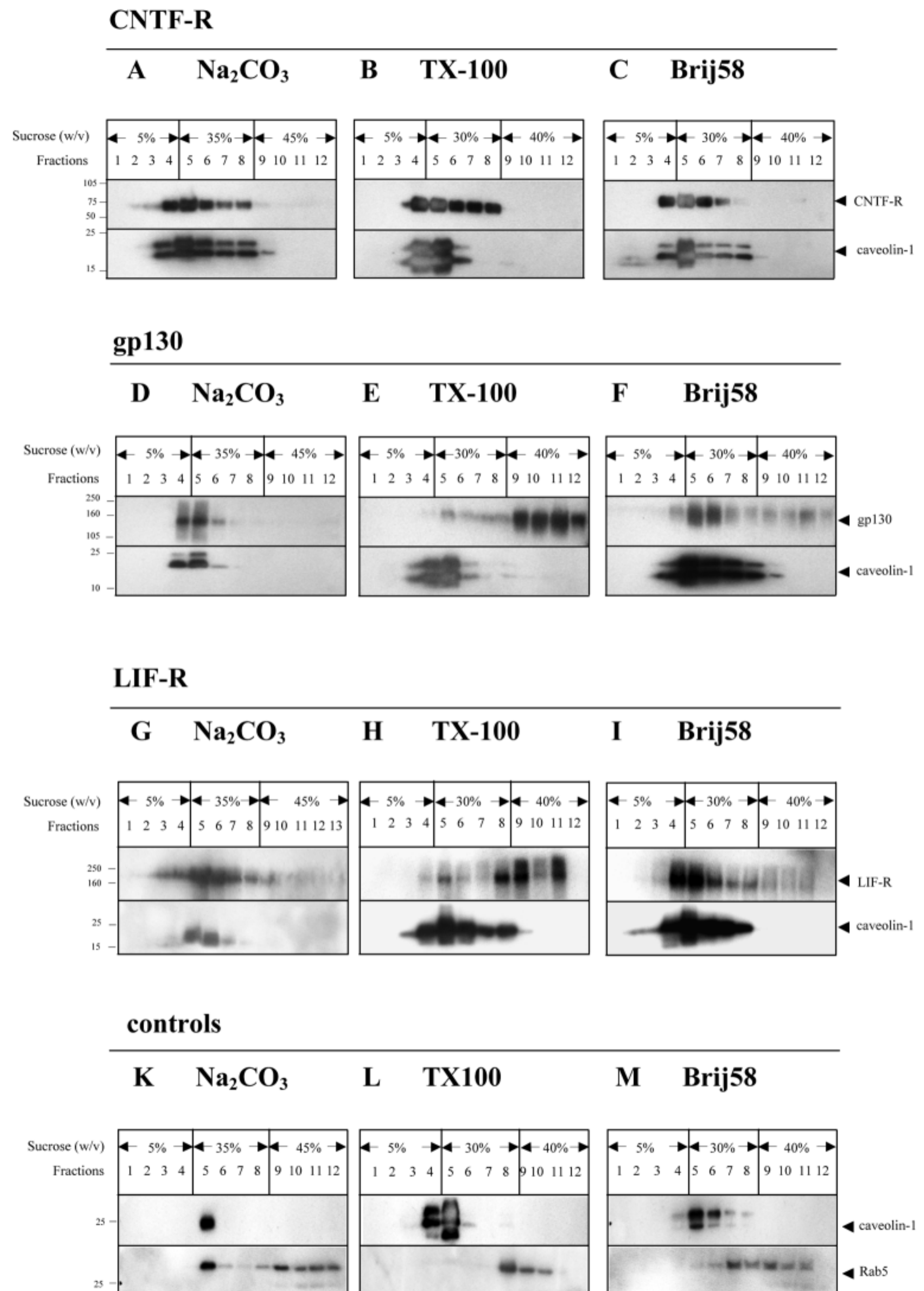

Fig. 5. Association with lipid rafts of human CNTF-R, human gp130, and canine LIF-R in stably transfected and parental MDCK cells. MDCK-CNTF-R (A-C), MDCK-gp130 (D-F), parental MDCK (G-I) and MDCK-CNTF-R (K-M) cells were grown on tissue culture dishes and lysed at 4°C using three different protocols: a detergent-free extraction with sodium carbonate buffer, pH 11 (A,D,G,K), extraction with 1% Triton X-100 (B,E,H,L), and extraction with 1% Brij 58 (C,F,I,M) (see Materials and Methods for details). Lysates were subsequently homogenised and adjusted to 45% (A,D,G,K) or 40% sucrose (B,C,E,F,H,I,L,M) respectively. Subcellular fractions were obtained by ultracentrifugation at 4°C in a sucrose gradient (45/35/5% or 40/30/5% sucrose) for 16-20 hours at 192,000 ***g*** in a Beckman SW40 rotor. 1 ml fractions were collected, TCA-precipitated and proteins were analysed by 10% SDS-PAGE. Proteins were transferred to a PVDF membrane and the CNTF-R (A-C), gp130 (D-F), or the LIF-R (G-I) and also the endogenous caveolin-1 (A-I) were sequentially detected with specific antibodies and HRP-conjugated secondary antibodies. In the control panels (K-M) blots were incubated with specific antibodies to Rab5 or caveolin-1 and respective HRP-conjugated secondary antibodies. The proteins were visualised using the ECL+plus system.

35% or 5-30%). After ultracentrifugation (39,000 rpm for 16-20 hours) fractions were collected, TCA-precipitated, and aliquots were analysed by SDS-PAGE and western blotting (Song et al., 1996). The membranes were sequentially incubated with antibodies against human CNTF-R (72 kDa) and caveolin-1 (24 kDa). Caveolin-1 is a scaffolding protein of caveolae (Okamoto et al., 1998; Scheiffele et al., 1998) – specialised invaginations at the basolateral plasma membrane with a lipid composition quite similar to lipid rafts – and it serves as a marker protein for this organelle (Song et al., 1996). As expected, endogenous caveolin-1 was detected in the lighter fractions four to eight with a peak at the interface between 5% and 30% or 35% sucrose. This is known to be the density at which lipid rafts and caveolar proteins float but which excludes most other cellular proteins (Song et al., 1996). Caveolin-1 migrated as a double band corresponding to the α- and the β-isoform between the 25 kDa and the 15 kDa molecular mass markers (Fig. 5A-M), which is in accordance with previous findings (Fujimoto et al., 2000). When the blot of the MDCK-CNTF-R cells was first developed with a CNTF-R-specific antibody a signal at about 70 kDa was detected in fractions four to eight (Fig. 5A-C) irrespective of the protocol used. As for caveolin-1, the strongest signal for CNTF-R was seen in fraction five corresponding to the 5-30%/35% sucrose

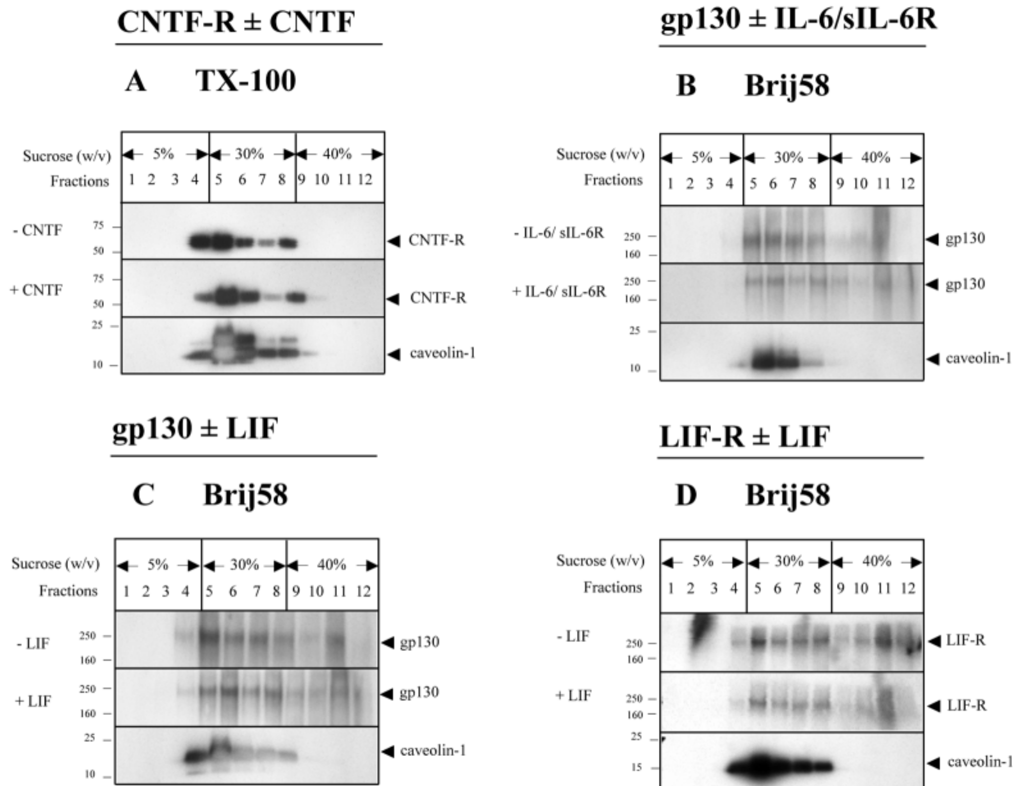

Fig. 6. Effect of receptor activation on lipid raft association. MDCK-CNTF-R cells (A), MDCK-gp130 (B,C), and parental MDCK cells (D) were stimulated with CNTF (A), IL-6/ sIL-6R (B) or LIF (C+D) for 30 minutes at 37°C and lysed at 4°C using the Triton X-100 (A) or the Brij 58 (B-D) protocol (see Materials and Methods). After ultracentrifugation in a 40/30/5% sucrose gradient at 4°C for 16-20 hours at 192,000 ***g*** in a SW40 rotor 1 ml fractions were collected, trichloracetic acid-precipitated, and proteins analysed by 10% SDS-PAGE and western blotting. Human CNTF-R (A), human gp130 (B,C), and the endogenous LIF-R (D), respectively, as well as endogenous caveolin-1 (A-D) were sequentially detected with specific antibodies and HRP-conjugated secondary antibodies. The proteins were visualised using the ECL+plus system.

interface. No immunoreactive protein was detected at the bottom of the gradient or the pellet, demonstrating that the CNTF-R is quantitatively associated with the floatable fractions. These results indicate that the CNTF-R is concentrated in lipid rafts.

It was of interest to know whether gp130 and LIF-R are also associated with lipid rafts. For gp130 this question was addressed in MDCK cells expressing human gp130 (Martens et al., 2000) because our antibodies do not detect canine gp130. In the case of the LIF-R, the endogenous receptor in parental MDCK cells was analysed. Whereas caveolin-1 showed the same flotation pattern in these cells with all three experimental protocols (Fig. 5D-I) the distribution of gp130 and LIF-R was dependent on the method used. Using sodium bicarbonate extraction both were quantitatively recovered from the fractions cofloating with caveolin-1 (Fig. 5D,G). By contrast, with the classic detergent extraction, using Triton X-100, gp130 and LIF-R were mainly found with the bulk cellular proteins at the bottom of the gradient. However, still a measurable portion (15-25%) of these receptors floated to the lighter fractions (Fig. 5E,H). When the detergent Brij 58 was employed about 65-85% of gp130 and LIF-R were floating with lipid rafts and about 15-35% were found at the bottom in heavy fractions (Fig. 5F,I). We also analysed the localisation of a bona fide non-raft marker, namely of the GTPase Rab5 (Fig. 5K-M). In both Triton X-100 and Brij 58, Rab5 did not float to a significant extent whereas with sodium bicarbonate extraction a significant proportion of Rab5 was found in the caveolin-1-containing fraction.

Because other receptors, such as the EGF-R, are known to move out of lipid rafts/caveolae upon stimulation (Mineo et al., 1999) we were interested whether stimulation alters the flotation of CNTF-R, gp130 or LIF-R. MDCK-CNTF-R cells were stimulated with CNTF (Fig. 6A), lysed, and analysed using the Triton X-100 protocol. No significant change in the flotation pattern of CNTF-R was observed upon cytokine treatment. For gp130 and LIF-R the respective experiments were performed using the Brij 58 method because under these conditions the majority of receptors float to lighter fractions (Fig. 5F,I). MDCK-gp130 cells were stimulated with either the IL-6/sIL-6 receptor complex or LIF for 30 minutes at 37°C (Fig. 6B,C). In addition, parental MDCK cells were stimulated with LIF and the LIF-R flotation was analysed (Fig. 6D). In this set of experiments no effect of receptor activation on their flotation behaviour was seen as well. Using the TX-100 protocol identical results were obtained (data not shown).

Cholesterol depletion abrogates CNTF-, LIF- and IL-6-induced STAT3 activation

The integrity of lipid rafts was disrupted by cholesterol depletion in order to assess whether association of components of the CNTF receptor complex with lipid rafts is necessary for activation of the Jak/STAT pathway by IL-6-type cytokines. Cells were preincubated with lovastatin/ mevalonate for 48 hours to reduce endogenous cholesterol production, followed by 10-20 mM methyl-β-cyclodextrin (MCD) for different times prior to stimulation (Hailstones et al., 1998; Rodal et al., 1999). Control cells were not preincubated with lovastatin and mevalonate. As shown in Fig. 7A,B, incubation with 10 mM MCD for 60 minutes resulted in a 39% reduction in the cellular cholesterol content, whereas with 20 mM MCD a 47% reduction was achieved.

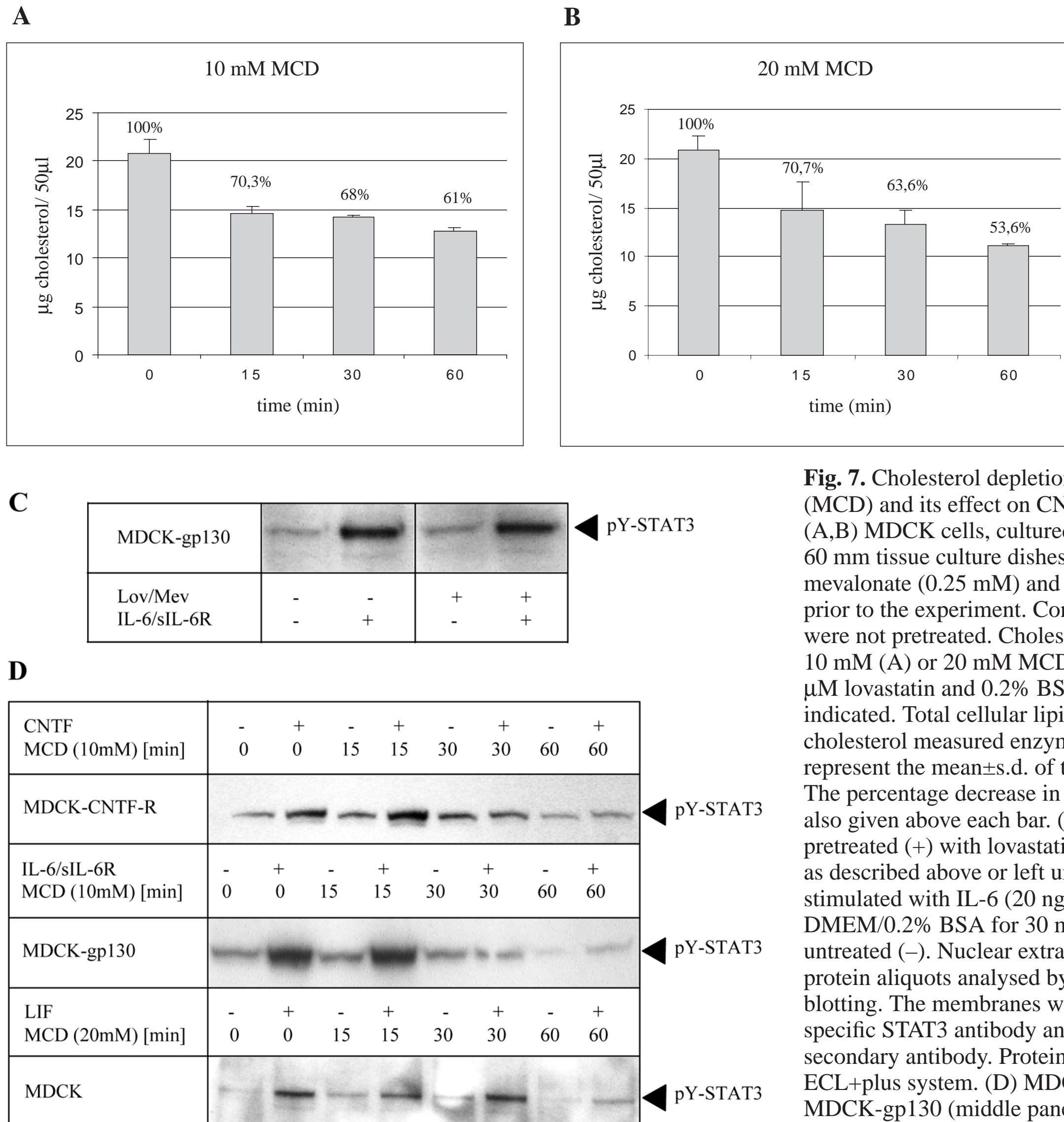

Fig. 7. Cholesterol depletion by methyl-β-cyclodextrin (MCD) and its effect on CNTF-, IL-6- and LIF-signalling. (A,B) MDCK cells, cultured for 72 hours, were plated on 60 mm tissue culture dishes and incubated with mevalonate (0.25 mM) and lovastatin (4 µM) for 48 hours prior to the experiment. Control cells (0 minutes MCD) were not pretreated. Cholesterol was depleted using 10 mM (A) or 20 mM MCD (B) in DMEM containing 4 µM lovastatin and 0.2% BSA at 37°C for different times as indicated. Total cellular lipids were extracted and cholesterol measured enzymatically at 500 nm. The data represent the mean±s.d. of three independent experiments. The percentage decrease in cellular cholesterol levels is also given above each bar. (C) MDCK-gp130 cells were pretreated (+) with lovastatin (Lov) and mevalonate (Mev) as described above or left untreated (–). The cells were stimulated with IL-6 (20 ng/ml)/ sIL-6R (500 ng/ml) in DMEM/0.2% BSA for 30 minutes at 37°C (+) or left untreated (–). Nuclear extracts were prepared and equal protein aliquots analysed by SDS-PAGE and western blotting. The membranes were probed with an activation-specific STAT3 antibody and an HRP-conjugated secondary antibody. Proteins were visualised using the ECL+plus system. (D) MDCK-CNTF-R (upper panel), MDCK-gp130 (middle panel) and parental MDCK cells (lower panel) were grown on tissue culture dishes and incubated with mevalonate and lovastatin as described above. Control cells (first two lanes) were left untreated. Lovastatin (4 µM) was also included in all depletion or stimulation solutions except for controls. The cells were cholesterol-depleted using 10-20 mM MCD at 37°C for different times as indicated and afterwards stimulated (+) with CNTF (25 ng/ml), IL-6 (20 ng/ml)/sIL-6R (500 ng/ml), or LIF (50 ng/ml), respectively. Nuclear extracts were prepared and analysed as outlined above.

Treatment with lovastatin/mevalonate alone resulted in a 25% reduction in cellular cholesterol (data not shown), however this had no influence on Jak/STAT activation by IL-6/sIL-6R (Fig. 7C) or other cytokines (data not shown). As shown in Fig. 7D (MDCK-CNTF-R, upper panel), 30-60 minutes preincubation of MDCK-CNTF-R cells with MCD strongly inhibited STAT3 activation by CNTF. Similar results were obtained with IL-6/ sIL-6R stimulation of MDCK-gp130 (MDCK-gp130, middle panel) and with LIF stimulation of parental MDCK cells (MDCK, bottom panel). This demonstrates that signalling of IL-6-type cytokines in MDCK cells is dependent on the cholesterol content of the cells and suggests that the integrity of lipid rafts is important for signal transduction. However, it is important to note that MCD treatment in no case led to redistribution of the studied receptors from the floating to the non-floating pool on sucrose gradients (data not shown).

Discussion

We have analysed the polarised expression and association with lipid rafts of the three components of the CNTF receptor complex, namely the CNTF-R itself and its signal transducers gp130 and LIF-R. One major finding of this study is that each receptor subunit has a distinct distribution in polarised epithelial cells. The preferential basolateral expression of gp130 has already been reported (Martens et al., 2000). For the human CNTF-R we find a predominant apical expression in stably transfected MDCK-CNTF-R cells (Fig. 1). This finding was not unexpected since for several other GPI-anchored

proteins an apical polarity has been shown before (Kollias et al., 1987; Lisanti et al., 1988; Powell et al., 1991; Wilson et al., 1990) leading to the assumption that a GPI-anchor acts as an apical sorting signal per se (Lisanti et al., 1989). However, recently at least one GPI-anchored protein, the scrapie protein PrP^C, was found at the basolateral domain of thyroid and kidney epithelial cells (Sarnataro et al., 2002). In contrast to the observed preference of the above described IL-6-type cytokine receptor subunits for either the apical or basolateral plasma membrane the LIF-R is obviously expressed at both sides to a significant level (Fig. 2C). This is an unusual finding since most receptors studied so far have shown a clear preference for one plasma membrane domain, usually the basolateral one (Beau et al., 1998; Maratos-Flier et al., 1987; Matter et al., 1994). A physiological relevance for an apical localisation of the LIF-R could lie in its role during blastocyst implantation in the uterus. Recently, LIF was found to be crucial for this process and is released by endometrial glands into the uterine lumen. LIF receptors were found in the luminal epithelium by in situ hybridisation and immunocytochemistry (Cheng et al., 2001). Although in this study an apical expression was not shown directly the ability of these cells to respond to luminal LIF strongly suggests that the LIF receptor must also be located on the apical side of luminal epithelial cells. Future studies will address this question directly.

Our results clearly demonstrate that MDCK cells express both LIF-R and gp130 endogenously suggesting that LIF might be a physiological regulator of kidney epithelial function. Barasch et al. reported that rat ureteric bud cells secrete LIF which then leads to the conversion of mesenchyme to epithelial cells during kidney development (Barasch et al., 1999). Furthermore, it was recently reported that in polarised hepatoma cells oncostatin M, another IL-6-type cytokine, which signals via a gp130/LIF-R heterodimer, stimulates bile canalicular membrane biogenesis (van der Wouden et al., 2002). This suggests that LIF-R/gp130 signalling is important for development of cell polarity.

For some proteins it was demonstrated that an apical sorting in MDCK cells corresponds to an axonal expression in neurons (Dotti et al., 1991). The subcellular localisation of the CNTF-R in authentic neuronal cells has not been determined so far which is probably because of the low receptor number in primary cells. However, a retrograde axonal transport of CNTF was demonstrated in adult sensory neurons suggesting that CNTF receptor components are expressed at the axonal membrane (Curtis et al., 1993). Two other neurotrophin receptors (TrkB, TrkC) were recently analysed in MDCK cells as well as in cultured hippocampal neurons (Kryl et al., 1999). Although one of them (TrkB) was expressed in MDCK cells in a polarised fashion (basolateral) both showed no evidence of vectorial sorting in neurons.

In stimulation assays on polarised MDCK cells we found that in MDCK-CNTF-R cells the Jak/STAT pathway can only be activated by CNTF added to the apical side (Fig. 4) which is consistent with the localisation of its binding subunit. Interestingly, LIF could strongly stimulate STAT3 activation from both cell surfaces. This implies that although gp130 is essentially a basolateral protein sufficient gp130 molecules must be present at the apical membrane to form functional CNTF and LIF receptor complexes, respectively. In fact, small amounts of gp130 were detected on the apical side of MDCK-gp130 cells with the biotinylation assay (Martens et al., 2000). We never observed a strong phosphorylation of STAT3 with apically added IL-6/sIL-6 receptors (Fig. 4) or via apically sorted truncated IL-6 receptors (Martens et al., 2000) suggesting that the majority of apical gp130 is not able to form functional complexes with IL-6 and the IL-6R. One possible explanation for this observation is that apical gp130 has already formed a preassociated complex with apical LIF-R and possibly also CNTF-R and therefore the formation of gp130 homodimers necessary for IL-6 signal transduction is not possible at the apical side. Both, LIF-R and CNTF-R are expected to be at a significant molar excess over gp130 at the apical surface. This explanation is not in accordance with the idea that the different receptor components of the IL-6-type cytokine family only associate after ligand binding. However, that notion is largely derived from coimmunoprecipitation or complex formation studies with purified extracellular domains of these receptors (Heinrich et al., 1998). Detergents used during immunoprecipitation may easily disrupt such preformed complexes. However, the transmembrane domain and its lipid environment may be crucial for the formation of these complexes. Lately, evidence is accumulating that members of the hematopoietic cytokine receptor family already form dimers without ligand binding and that a ligand triggers a conformational change within such a dimer resulting in its activation. Such a scenario was recently proposed for the erythropoietin receptor (Frank, 2002). For the interferon-γ (IFN) receptor a preassembly of the IFN-γ receptor chains was also observed (Krause et al., 2002). The extracellular part of the IL-6R has recently been show by X-ray analysis to be an IL-6R dimer prior to IL-6 binding (Varghese et al., 2002). We and others are currently testing whether this is also the case for other IL-6-type cytokine receptors.

GPI-anchored proteins seem to promote their apical localisation through association with lipid rafts (Simons and Ikonen, 1997). This was shown for several GPI-anchored proteins (Schroeder et al., 1998) by means of a specific fractionation assay (Brown and Rose, 1992). We could show by fractionation of MDCK-CNTF-R cells in a sucrose density gradient using different protocols (detergent-free with Na_2CO_3, TX-100, Brij 58) that the CNTF-R elutes exclusively in fractions typical of lipid raft components (Fig. 5). The association with lipid rafts was independent of the absence or presence of a ligand (Fig. 6). This suggests that the ternary CNTF receptor complex is localised to and signals from lipid rafts. In fact both, gp130 and the LIF-R could also be detected in the floatable fraction (Fig. 5). However, in contrast to the CNTF-R, significant amounts of both proteins were also found in the bottom fractions indicating the existence of a non-raft pool of gp130 and LIF-R. Since the partial association of gp130 and LIF-R with lipid rafts was observed in cells not expressing the CNTF-R the localisation to rafts must be a genuine property of each receptor. Whether this is due to a posttranslational modification such as palmitoylation is currently under investigation.

The percentage of gp130 and LIF-R found to be associated with lipid rafts was highly dependent on the methodology used for isolation. With sodium bicarbonate essentially all gp130 and LIF-R floated to lighter caveolin-1-containing fractions. However, under these conditions also substantial amounts of Rab5, a non-raft marker, were found at the 5%/35% interface.

This indicates that at least in MDCK cells the sodium carbonate extraction is not useful to assess lipid raft association of proteins. With Brij 58 extraction about 65-85% of gp130 and LIF-R were associated with lipid rafts. When using the classic Triton X-100 protocol, about 15-25% of the receptors floated to fractions with lighter densities. Under this regimen Rab5 was completely detergent-soluble. A similar difference in detergent extractability was also reported for the EGF-R. In the case of gp130 and LIF-R no changes in the distribution of the receptors were observed after stimulation with either IL-6/sIL-6R or LIF regardless of whether the Brij 58 (Fig. 6) or the Triton X-100 protocol (data not shown) was used.

Schuck et al. recently analysed detergent-resistant membrane (DRM) fractions isolated with different detergents (including Brij 58 and Triton X-100) and found considerable differences in their protein and lipid composition (Schuck et al., 2003). In this study, Triton X-100 was found to be the most specific detergent in respect of the enrichment of sphingolipids and cholesterol and to the separation of raft and non-raft proteins. Whether the differential insolubility of proteins in certain detergents reflects the existence of different subtypes of lipid rafts as suggested by Röper et al. (Roper et al., 2000) or the differential strength of protein/lipid raft interactions (Schuck et al., 2003) is currently unclear. Alternative tools like fluorescence spectroscopy techniques are needed to solve this question in the future.

Neuronal cells also contain lipid rafts with which GPI-anchored proteins associate (Krämer et al., 1997; Madore et al., 1999; Olive et al., 1995). Since the signal transduction of the CNTF receptor complex in the MDCK model takes place at the apical membrane and the α-receptor is associated with lipid rafts it is likely that the CNTF-R is also concentrated in lipid rafts of neuronal cells and signals in these domains. In neuronal PC12 cells both the nerve growth factor (NGF) receptor $p75^{NTR}$ and the receptor tyrosine kinase TrkA were shown to signal from caveolae-like membrane domains (Huang et al., 1999).

In the case of the signal transducer gp130 an association with lipid rafts had already been proposed (Koshelnick et al., 1997). However, this was an untypical receptor complex since it involved the GPI-linked urokinase receptor. In multiple myeloma and Hep3B cells gp130 was also found to be associated with lipid rafts/caveolae isolated by detergent-free methods (Podar et al., 2003; Sehgal et al., 2002). In both cases signalling via gp130 was found to be sensitive to cholesterol depletion by MCD. Our result that IL-6/sIL-6R signalling is markedly inhibited by MCD confirms these data (Fig. 7). In our case, also STAT3 activation by CNTF and LIF was sensitive to MCD treatment, suggesting that signalling via these heterodimeric receptor complexes is likewise dependent on raft integrity. Surprisingly, none of the receptor subunits moved from the floating pool to non-raft fractions upon MCD extraction (data not shown) indicating that in MDCK cells detergent-resistant rafts are unusually stable. This notion is supported by the findings of Schuck et al. showing that in MDCK cells even cholesterol depletion by 70% did not disturb raft association of several marker proteins unless a cell homogenate was used (Schuck et al., 2003). Since in our case receptors do not redistribute upon MCD treatment one has to assume that other critical signalling components may be affected by cholesterol depletion.

Nevertheless, our results suggest that lipid raft integrity is important in CNTF-, LIF- and IL-6-induced Jak/STAT activation. For the IL-2 receptor complex in T-cells it was recently found that the α-subunit is also raft-associated. However, the signal transducers IL-2Rβ and IL-2Rγ as well as Jak1 and Jak3 were found in soluble membrane fractions. IL-2-mediated receptor complex formation occurred within soluble non-raft membrane fractions and IL-2 signalling was not sensitive to methyl-β-cyclodextrin (Marmor and Julius, 2001). In this case lipid rafts acted as negative regulators of IL-2 signalling by sequestering the α-chain. A similar inhibitory role of lipid rafts was recently proposed for the EGF-R (Roepstorff et al., 2002). In the IL-6-type cytokine receptor system such a negative role of lipid rafts is unlikely since the CNTF-R is completely associated with rafts even upon stimulation. Whether and how raft association is linked to the differential sorting of the studied receptor complex components is an open question and currently under investigation.

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