# PrP-dependent cell adhesion in N2a neuroblastoma cells

Alain Mangé[a], Ollivier Milhavet[b], David Umlauf[a], David Harris[c], Sylvain Lehmann[a,*]

[a] *Institut de Génétique Humaine, CNRS U.P.R. 1142, 141, rue de la Cardonille, 34396 Montpellier Cedex 5, France*
[b] *National Institutes of Health, National Institute on Aging, Gerontology Research Center, Laboratory of Neurosciences, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA*
[c] *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110-1093, USA*

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**Abstract The cellular isoform of prion protein ($PrP^C$) is a ubiquitous glycoprotein expressed by most tissues and with a biological function yet to be determined. Here, we have used a neuroblastoma cell model to investigate the involvement of PrP in cell adhesion. Incubation of single cell suspension induced cell–cell adhesion and formation of cell aggregates. Interestingly, cells overexpressing PrP exhibit increased cation-independent aggregation. Aggregation was reduced after phosphatidylinositol-specific phospholipase C release of the protein and by preincubation of cells with an antibody raised against the N-terminal part of $PrP^C$. Our paradigm allows the study of the function of PrP as an intercellular adhesion molecule and a cell surface ligand or receptor.** 

*Key words:* Aggregation; Intercellular adhesion; Prion protein

## 1. Introduction

The agent responsible for the transmissible spongiform encephalopathies (TSEs) is thought to be a protein called $PrP^{Sc}$ (scrapie isoform of prion protein) which represents a conformational variant of the cellular isoform of the prion protein ($PrP^C$) [1]. $PrP^C$ is a ubiquitous glycoprotein expressed particularly at high levels by neuronal cells [2]. Its normal function remains uncertain, although its localization on the cell surface would be consistent with roles in cell adhesion, ligand uptake or transmembrane signaling [3]. It has been also proposed that $PrP^C$ could act during development, as its expression is detectable early in embryogenesis and increases during development [4,5].

Several GPI-anchored proteins are implicated in cell–cell adhesion mechanisms, such as the $Ca^{2+}$-independent neural cell adhesion molecule (N-CAM). Data reported recently are consistent with a possible function of PrP as a cell adhesion or recognition molecule. In particular, the binding between PrP and putative receptor or ligand was recently demonstrated. In these studies, the 67-kDa laminin receptor (LR), the 37-kDa laminin receptor precursor (LRP) and the extracellular matrix glycoprotein laminin seem to interact with PrP [6–8]. Laminin, through its cell membrane receptors, is known to play a pivotal role in cell proliferation, neuronal death and differentiation [9,10]. Thus, it was proposed that the specific interaction between PrP and laminin, LR or LRP could have consequences for neurite outgrowth, neuronal survival and differentiation via an unknown signaling event [6,7,11]. A signal transduction pathway associated with PrP has been partially characterized and occurs via the tyrosine kinase $p59^{fyn}$ in the fully differentiated neuronal cells [12]. The $p59^{fyn}$ kinase has been implicated in N-CAM-induced neurite outgrowth in rat PC12 cells [13]. Interestingly, it has been postulated recently that N-CAMs are also good candidates to interact with $PrP^C$ in mouse neuroblastoma N2a cells [14]. Taken together, these data suggested that $PrP^C$ may act as a cell surface ligand or receptor mediating cell signaling.

Herein, we demonstrate that PrP is involved in a cell adhesion process that leads to intercellular aggregation. The cell–cell adhesion mediated by PrP is cation-independent and is inhibited by an antibody against the N-terminal part of PrP or by treatment of cells with phosphatidylinositol-specific phospholipase C (PIPLC).

## 2. Materials and methods

### 2.1. Reagent and antibodies

Opti-modified Eagle's medium (MEM), trypsin, geneticin, Lipofectamine were from Life Technologies Inc. (Cergy-Pontoise, France) and fetal calf serum from Bio-Whitaker (Fontenay-sous-bois, France). PIPLC was purchased from Roche Diagnostics (Meylan, France). All other reagents were from Sigma (St Quentin Fallavier, France).

Rabbit polyclonal antibody P45–66, raised against the synthetic peptide encompassing moPrP residues 45–66 (CGGNRYPPQGGTWGQPHGGGWGQ), has been described earlier [15]. In some experiments, the antibody P45–66 was blocked by incubation with its specific peptide for 30 min before use. Secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA).

### 2.2. N2a cell cultures

The mouse neuroblastoma cell line N2a was stably transfected with wild-type mouse *Prnp-a* cDNAs without (N2a #58 clone) or with (N2a #37 clone) a 3F4 tag, as previously described [15]. Transfected and untransfected N2a cells were grown in Opti-MEM containing 10% fetal calf serum and penicillin/streptomycin in an atmosphere of 5% $CO_2$/95% air. In some experiments, N2a cells were transiently transfected with a cDNA encoding wild-type moPrP using Lipofectamine as recommended by the manufacturer, and used 48 h after transfection.

### 2.3. Western blot

For Western blotting, cell lysates were electrophoresed on a 12% sodium dodecyl sulfate (SDS)/glycine/polyacrylamide gel. Proteins

*Corresponding author. Fax: (33)-4 99 61 99 01.
*E-mail address:* sylvain.lehmann@igh.cnrs.fr (S. Lehmann).

*Abbreviations:* PrP, prion protein; $PrP^C$, cellular isoform of PrP; $PrP^{Sc}$, scrapie isoform of PrP; PIPLC, phosphatidylinositol-specific phospholipase C; LR, laminin receptor; LRP, laminin receptor precursor; N-CAM, neural cell adhesion molecule

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were electroblotted onto Immobilon-P membranes and PrP was detected by using the antibody P45–66 and a peroxidase-conjugated goat anti-rabbit secondary antibody. The blots were developed using enhanced chemiluminescence (ECL).

*2.4. Cell aggregation assay*

Confluent transfected or untransfected N2a cells were lightly trypsinized (0.01%) for 5 min. The level of $PrP^C$ was not significantly affected after this treatment (data not shown). Single cell suspensions were obtained by passing cells through a G23 gauge needle. Suspensions were adjusted to a final concentration of $10^6$ cells/ml in Opti-MEM containing 5% fetal calf serum (FCS) and 5 μg/ml DNase I. Cells were seeded in 24-well plates and allowed to aggregate on a rotary shaker at 90–100 rpm. Cell viability was checked by using a trypan blue assay. More than 95% of the cells remain alive throughout the procedure. After 40 min of agitation, cells were fixed by the addition (v/v) of 4% paraformaldehyde, 0.1% glutaraldehyde in phosphate-buffered saline (PBS). This fixation prevents the dissociation of the aggregates and is responsible for the shrunken shape of the cells under phase contrast. For quantitation of aggregation, cells were carefully resuspended and single cells were counted under phase contrast microscopy. Aggregation is defined as the ratio of single cells to the total number of cells counted. A minimum of 600 cells was counted in triplicate for each experiment. In some cases, inhibition assays were conducted by adding to the cell suspension antibodies or metal chelators.

*2.5. PIPLC treatment*

Confluent cells were rinsed with cold PBS, incubated for 2 h on ice with the bacterial enzyme PIPLC (1 unit/ml in Opti-MEM) which cleaves the diacylglycerol portion of the glycosyl-phosphatidylinositol anchor, and then cell aggregation assays were performed as described previously.

*2.6. Statistical analysis*

Comparisons were made with analysis of variance, followed by the unpaired *t*-test. A value of $P < 0.05$ was considered statistically significant. Where *t*-test shows a significant difference, this is indicated by an asterisk.

## 3. Results and discussion

$PrP^C$ is a highly conserved cell surface glycoprotein expressed by a broad range of cells and in particular by neuronal cells [2]. The normal function of $PrP^C$ is still uncertain but may be related to metal ion transport or cellular response to oxidative stress. In addition, due to its localization on the cell surface, it has been suggested that $PrP^C$ might function as a receptor or an adhesion molecule [3]. In an attempt to evaluate a putative role of $PrP^C$ in cell adhesion, we compared the aggregation of single cells in suspension that expressed different levels of $PrP^C$.

We used the mouse neuroblastoma cell line N2a that endogenously expresses $PrP^C$ on its surface (Fig. 1A, lane 1) [16]. We transfected these cells to express high levels of $PrP^C$ and then measured their ability to aggregate. The levels of $PrP^C$ expressed in untransfected N2a and transfected N2a subclones were compared by immunoblotting with P45–66 antibody (Fig. 1A). Quantification of the signal revealed that N2a clones #58 and #37 have four times the PrP level of untransfected N2a cells. We assayed these different cell lines for aggregation as described in Section 2. As shown in Fig. 1B, the two N2a subclones overexpressing PrP showed an increased aggregation in comparison with the untransfected cells. The cell lines overexpressing PrP usually gave larger cellular aggregates than untransfected cell lines (Fig. 1B). After quantification, we observed that overexpression of PrP in the two N2a subclones #58 and #37 increased the cell aggregation by approximately 30% compared to the untransfected cells (Fig.

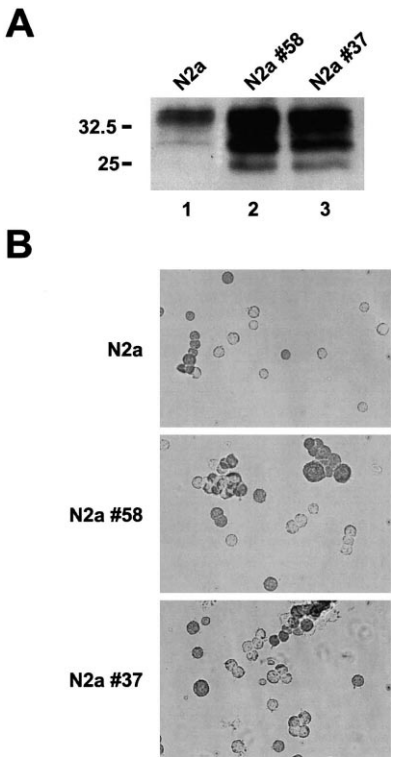


Fig. 1. Detection of $PrP^C$ in different cell lines, and light microscopic observation of cell aggregation. A: Cell lysates were analyzed by Western blotting with the PrP N-terminal specific antibody P45–66. N2a cells (lane 1) were stably transfected with mouse PrP. Two subclones of stably transfected cells were analyzed: subclone N2a #58 transfected with moPrP (lane 2) and subclone N2a #37 transfected with 3F4-tagged moPrP (lane 3). The position of molecular size marker protein is in kDa. B: The cultured cells were released from the culture dish, incubated in a standard aggregation assay, fixed and then photographed at low magnification. The shrunken aspect of the cells is a consequence of the fixation procedure. Untransfected N2a cells (upper panel) aggregated less than transfected N2a #58 cells (middle panel) or N2a #37 cells (lower panel).

2A). Cell aggregation was also increased (by 34%) when N2a cells were transiently transfected which result in an overexpression of $PrP^C$ comparable to that of stably transfected cells. These findings obtained in two different clones and in a population of transiently transfected cells demonstrate that $PrP^C$ promotes aggregation of N2a cells.

In order to confirm that $PrP^C$ on the cell surface participates in the cell aggregation observed here, aggregation was assayed with untransfected N2a cells and transfected N2a cells (clone #58) which were pretreated with PIPLC to remove cell surface PrP (Fig. 2B). Release of $PrP^C$ was verified by Western blotting analysis (data not shown). The pre-incubation of N2a clone #58 cells with PIPLC reduced the formation of cell aggregates to the level of that of the untransfected N2a cells (Fig. 2B). Interestingly, the aggregation of untransfected N2a cells seems not be affected by PIPLC treatment. This suggests that PrP expressed at an endogenous level is not the main cell adhesion molecule in these cells. We demonstrated the specificity of the aggregation by treatment with an anti-PrP antibody (Fig. 3A). Treatment of N2a #37 cells with P45–66, a polyclonal antibody raised against the N-terminal part of PrP, significantly inhibits cell aggregation compared to the same cells treated with the corresponding pre-immune sera or

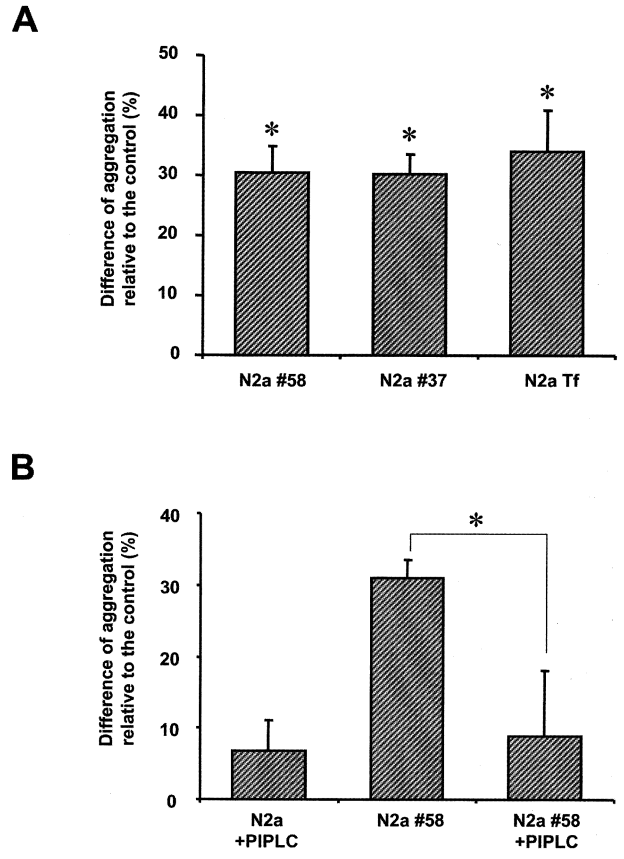


Fig. 2. Quantitative analysis of N2a cell aggregation. A: Aggregation of untransfected or transfected N2a cells was assayed as described in Section 2. Aggregation is expressed as the difference in percent of the aggregation of the cell lines calculated relative to that of untransfected N2a cells (control). Each bar represents the mean ± the standard deviation from at least three independent experiments. Asterisks indicate significant differences between control and transfected cells ($P < 0.05$). B: Prior the aggregation assay, N2a and N2a #58 cells were incubated in presence or absence of PIPLC. Each bar represents the mean aggregation relative to untreated N2a cells from three experiments. Asterisks indicate a significant difference between untreated and PIPLC-treated N2a #58 cells ($*P < 0.05$).

with the antibody P44–66 blocked by pre-incubation with its specific peptide. These data suggest that the N-terminal part of PrP plays a role in the aggregation phenomenon. Taken together, our results indicate that PrP could function as an adhesion molecule responsible for increasing the aggregation of PrP-overexpressing N2a cells.

Most molecules involved in cell adhesion can be classified into three different families: the integrins, the cadherins and the immunoglobulins superfamily (for reviews see [17,18]). Most of these molecules mediate adhesion in a cation-dependent manner. $Ca^{2+}$ and $Mg^{2+}$ in particular are involved in these phenomena. $PrP^C$ is known to bind copper through its octapeptide region in the N-terminal half of the protein [19]. More data are needed to confirm the relation between $PrP^C$ and copper, but this ion seems to be involved both in the acquisition of the conformation of the N-terminus of $PrP^C$ through its binding to the repeat region [20,21] and in the function of the $PrP^C$ [22–24]. To investigate a possible role of metal ions in $PrP^C$-mediated aggregation, we examined the effect of chelators on aggregation. As shown in Fig. 3B, addition of copper chelators (DTPA, neocuproin or bathocuproin) or a cation chelator (EDTA) during the assay had no significant effect, indicating that $PrP^C$-mediated adhesion occurs in a cation-independent manner.

The results presented here show that when PrP-overexpressing N2a cells are in suspension, a specific intercellular adhesion occurs through cell surface PrP. This cell–cell adhesion is cation-independent and is inhibited by an antibody against the N-terminal part of PrP or by treatment of cells with PIPLC. The cell aggregation is most likely mediated by the specific transcellular binding of $PrP^C$ on the surface of one cell with an unknown molecule on the surface of the other. Many studies have been conducted to identify $PrP^C$-binding proteins. Among the putative candidates, $PrP^C$ seems to interact with the 37-kDa LRP and the 67-kDa LR [7,8], the extracellular matrix glycoprotein laminin [6] and very recently with N-CAMs [14]. All these data are consistent with a possible function of $PrP^C$ as a neuronal receptor and cell adhesion or recognition molecule. Many putative N-CAMs have been implicated in different developmental and cellular processes in-

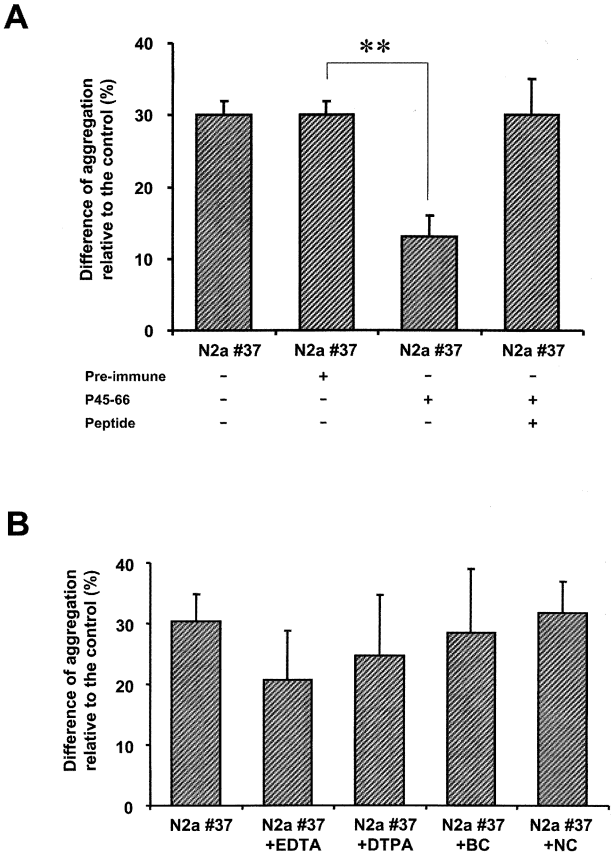


Fig. 3. Quantitative analysis of aggregation in presence or absence of antibodies or metal ion chelators. A: Aggregation of N2a #37 cells was performed in the absence of antiserum or in the presence of pre-immune sera at 1/50, P45–66 polyclonal antibody at 1/50 or P45–66 antibody blocked with its specific peptide. B: Aggregation of N2a #37 cells was performed in absence of chelator or in the presence of 2 mM EDTA, 1 mM DTPA, 100 μM bathocuproin (BC) or 100 μM neocuproin (NC). The amount of cell aggregation with or without treatment was plotted. Each bar represents the mean aggregation relative to untreated cells from three experiments. Asterisks indicate significant differences between untreated and treated cells ($**P < 0.01$).

cluding neurite outgrowth, neuronal cell migration, cell-to-cell interaction or cell signaling (for reviews see [17,18]). Interestingly, the PrP/laminin interaction seems to be important for the neuritogenesis of cultured hippocampal neurons [6] and for the differentiation and neurite outgrowth of PC12 cells [11]. Moreover, binding of $PrP^C$ to the LRP/LR receptor mediates the internalization of $PrP^C$, a process which could be linked to signal transduction events [7]. One could imagine that the specific intercellular adhesion mediated by $PrP^C$ in our aggregation model could result in activation of an intracellular signaling process similar to that observed in other adhesion events [13,25]. In particular, $PrP^C$ has been implicated recently in signal transduction activity via activation of the tyrosine kinase $p59^{fyn}$ [12]. Further experiments are planed to identify a possible signal transduction response after cell–cell adhesion as shown in another model [26]. An additional issue concerns the domain of PrP that is involved in adhesion. Using antibodies as blocking agents in the aggregation assay, we have shown that the N-terminal part of PrP containing the octapeptide region is involved. In addition to binding copper, this region also seems to bind heparin sulfate proteoglycans (HSPGs) [27,28]. HSPGs are widely distributed in mammalian tissues and play an essential role during neural development (for review see [29]). Interestingly, it has been postulated that HSPGs act as co-factors for PrP/LRP binding through a domain that encompasses residues 53–93 [30]. In conclusion, our results point out the possible role of $PrP^C$ in cell adhesion and recognition mechanisms. The intercellular aggregation mediated by PrP in our model could provide insight into the interactions between $PrP^C$ and its putative receptors, and to identify the phenotypic modifications triggered by this binding. These insights would contribute to a better understanding of the role of normal and pathological PrP molecules.

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