ROLE OF 4F2HC IN TUMORIGENESIS

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1. Summary of the project

4F2hc (CD98hc, SLC3A2, FRP1) is a multifunctional protein involved in cell transformation, integrin signaling, cell fusion and amino acid transport. 4F2hc is a type II glycoprotein, which forms heterodimers with different light subunits. We solved the atomic structure of the ectodomain of 4F2hc (4F2hc-ED). This domain contains a TIM barrel and an all \( \beta \)8 antiparallel subdomain. The cleft of 4F2hc-ED is wider and deeper than in homologous glycosidases.

The main objectives of this project are: 1) impact of 4F2hc repression in proliferation, tumor formation and potential metastatic activity of tumor cell lines. 2) Identification of ligands of 4F2hc using glycan-chips, docking in silico and proteomic approaches. 3) If new 4F2hc ligands are identified their impact on 4F2hc functions will be analyzed. 4) Role of 4F2hc-ED, using a mutated version of the protein, in cellular systems on amino acid transport, integrin signaling, cell transformation and tumor formation.

Demonstrating the role of 4F2hc in tumor formation and the identification of natural and synthetic ligands of 4F2hc might add a new therapeutic target for cancer treatment, and the generation of the first tools towards this goal.

2. Results

4F2hc (the heavy chain of the cell surface antigen complex 4F2; also named CD98 or fusion regulatory protein-1; FRP1) is a multifunctional protein involved in amino acid transport, \( \beta \)-integrin signaling, cell fusion and tumorigenesis (Chillarón et al., 2001; Cantor et al., 2008). 4F2hc is a type II membrane glycoprotein (i.e., intracellular N-termini, one trans-membrane segment and a large N-glycosylated ectodomain).
4F2hc is linked by a disulphide bridge involving residue Cys109 (human protein numbering) with one of six light subunits in humans (LAT1, LAT2, y+LAT1, y+LAT2, asc1 and xCT) (Palacín et al., 2005). The light subunits are the corresponding amino acid transporters (Reig et al., 2002). Thus, the six 4F2hc heterodimers have different amino acid exchanger activities (two isoforms of exchanger L (LAT1 and LAT2); two isoforms of exchanger y+L (y+LAT1 and y+LAT2), exchanger asc (asc1) and exchanger x_c^- (xCT). 4F2hc is needed to bring the holotransporter to the plasma membrane (Chillarón et al., 2001).

4F2hc also mediates β-integrin signaling and cell spreading (Feral et al., 2005; 2007). It has been reported that this interaction is mediated by intracellular and trans-membrane domains of 4F2hc and the intracellular domain of β-integrins (Prager et al., 2007). Similarly, by studding 4F2hc-CD69 chimeras, it has been suggested that the same 4F2hc domains are essential for the ability of embryonic stem cells to produce teratocarcinomas in nude mice (Feral et al., 2005). However, in these experiments not all possible chimeras have been studied leaving room for a role of the ectodomain of 4F2hc in these functions. Similarly, the mutant Cys330Ser located in the ectodomain of 4F2hc is a dominant negative mutant that blocks cell fusion induced by 4F2hc antibodies (Okamoto et al., 1997). Thus, the role of the ectodomain of 4F2hc in the multiple functions of the protein is not well established.

In this project we proposed to study the role of the ectodomain of 4F2hc in the multiple functions of the protein with the focus on amino acid transport, β-integrin function and tumorigenesis. At the beginning of the project we solved the atomic structure of the ectodomain of human 4F2hc (4F2hc-ED) a 2.1 Å. One of the crystal structures solved revealed homodimers of 4F2hc-ED (Fort et al., 2007). Below are described our results on the homodimerization of 4F2hc and the role of the ectodomain of 4F2hc amino acid transport, β-integrin function and tumorigenesis.
**Homodimerization of 4F2hc**

The atomic structure of the ectodomain of human 4F2hc (4F2hc-ED) solved in a particular set of 3D crystals revealed homodimers of 4F2hc in the asymmetric unit. The homodimer presents a contact surface on each subunit of approx. 1200 Å with 10 hydrogen bonds and a salt bridge between the two subunits. In addition, an atom of Zn$^{2+}$ is coordinated between both subunits at residues Asp439, His441 and His455 of monomer 1 and His441 of monomer 2 (Fort et al., 2007). In contrast, 4F2hc-ED is a monomer in solution (Turnay et al., submitted). To check if homodimerization of 4F2hc was an artefact of the crystal, several experiments in cells were conducted to demonstrate the formation of 4F2hc homodimers in cell culture.

HeLa cells were cotransfected with two tagged versions of human 4F2hc (4F2hc-HA, HA tag at the C terminus; His-4F2hc, six His tag at the N terminus). After His affinity purification, an HA-immunodetected high molecular weight band (170–185 kDa) was observed only in cells cotransfected with both 4F2hc-tagged versions. This indicated the formation of 4F2hc homodimers. Human 4F2hc homodimers are formed in the endoplasmic reticulum (ER) as demonstrated by its sensitivity to endo H treatment. Surface biotin labeling revealed expression of 4F2hc-HA monomers and homodimers at the plasma membrane. 4F2hc homodimers were abolished in reducing conditions. Human 4F2hc expressed in CHO-K1 cells or in Xenopus oocytes also showed DTT-sensitive bands with SDS-PAGE mobility compatible with the formation of homodimers. Mutation Cys109Ser abolished the presence of 4F2hc homodimers in SDS-PAGE, demonstrating that Cys109 is the residue forming the disulphide bridge between the two 4F2hc monomers (Fort et al., 2007). Interestingly, mutation C109S did not abolish 4F2hc homodimer formation detected by co-immunoprecipitation. This indicated the tendency to homodimerize of 4F2hc besides disulfide bridge formation. Surprisingly, mutations that ablate the Zn$^{2+}$ coordination patch (D439A-H441A-H455A), observed in 4F2hc 3D crystals, did not abolish the formation of 4F2hc.
homodimers. In summary, our results demonstrated that overexpression of human 4F2hc in cultured cells results in the formation of 4F2hc homodimers linked by a disulphide bridge through residue Cys109 and expressed at the cell surface (Fort et al., 2007).

Cross-linking experiments were used to check the geometry of 4F2hc homodimerization in cell culture. BM[PEO]₂, a reagent that is membrane-impermeable and noncleavable by reducing agents with the capacity to crosslink thiol groups 3.5–14.7 Å apart, was used. Serines 444 and 480 are located in 4F2hc-ED at a distance to be cross-linked by the reagent in the crystal homodimer. Mutations to Cys were introduced in these residues and Cys109 was mutated to Ser to check cross-linking between Ser444 (mutant C109S-S444C) or Ser480 (mutant C109S-S480C) between the two monomers. SDS-PAGE in the presence of DTT demonstrated the presence of 4F2hc homodimers after cross-linking with BM[PEO]₂. Finally, protein-protein docking simulations in the absence of Zn²⁺ coordination by taking two copies of the monomeric structure of 4F2hc-ED (PDB code 2DH3) were performed. The second-best docking solution was in agreement with the symmetry in the crystal homodimer and distance restrictions for cross-linking experiments and Cys109 disulfide bridge formation. Indeed, this solution is very similar to the obtained crystal structure. Thus, over-expression of 4F2hc in cells resulted in the formation of 4F2hc homodimers with geometry very similar, if not identical, to the solved crystal structure, implying the formation of a disulphide bridge between residue Cys109 in each monomer (Fort et al., 2007).

Calculations also suggested that homodimerization is guided by shape and electrostatic complementarities rather than by the hydrophobic effect. These studies allowed us to propose a structural model for the homodimer of 4F2hc. The restrictions imposed by the homodimer geometry position one of the sides of the TIM barrel (domain A) of 4F2hc-ED towards the external surface of the plasma membrane (Fort et al., 2007). Molecular dynamics analysis of 4F2hc-ED homodimer supports an electrostatic interaction of this ectodomain with
the polar moiety of membrane phospholipids at the cell surface (García, Orozco, Palacín; unpublished results).

**Over-expression of 4F2hc in tumors**

Screening of paraffin-embedded tissues from the tumor bank of the Hospital Clínic in Barcelona (Dr J.A. Fernández) with anti-4F2hc 4F2 (Prof. Francisco Sánchez-Madrid) revealed over-expression of 4F2hc in several human tumors (colon adenocarcinoma, salivary gland adenosquamous carcinoma, stomach adenocarcinoma (non-differentiated), hepatocarcinoma (non-differentiated)). Particularly relevant is the over-expression of 4F2hc in colorectal cancer. Indeed, 17 out 18 independent colon adenocarcinoma samples (including low-grade, moderate-grade and metastatic high-grade stages) showed overexpression of 4F2hc, both detected by immunohistochemistry and western blot (unpublished results). 4F2hc heterodimer over-expression was found in the analyzed tumor samples, with no presence of 4F2hc monomers and homodimers, suggesting strong co-regulation of the expression of 4F2hc and its light subunits (Burghardt, Fernández, Palacín; unpublished results). Several tumor samples and cell lines (e.g., HeLa, HEK293T) express different forms of 4F2hc revealed by EndoF treatment. Some of these 4F2hc immunoreactive bands have a higher molecular weight than the bona fide 4F2hc protein, suggesting alternative splicing (Burghardt, Fernández, Palacín; unpublished results).

Next we analyzed the expression of 4F2hc in a panel of human colorectal cancer cells, the non-tumorigenic BCS-TC2 cells and the tumorigenic BCS-TC2.2, BCS-TC2.BR2, BCS-TC2.FN, Caco-2 and Colo-320 cells, among others. In addition, the expression of 4F2hc in cells from other types of human tumors (HeLa, OVCAR, A431 and RD cells) was also analyzed. Different levels of expression and glycosylation of 4F2hc were detected among the different cells. Two of these cell lines, BCS-TC2.2 and BCS-TC2.BR2, were selected for tumor formation studies in nude mice. We have not observed a direct relationship between the level of 4F2hc expression and extent of glycosylation with the
tumorigenicity of the cells. Treatment of cells with tunicamycin, which inhibits glycosylation of newly-synthesized proteins, and/or with EndoF yields a major unglycosylated band. In HeLa cells, but not in human colon adenocarcinoma cells, two lower mobility bands are also detected (Turnay, Lizarbe; unpublished results). Transformation of mammalian cells (e.g., CHO) to produce tumors in nude mice is dependent on 4F2hc over-expression upon transfection (Henderson et al., 2004). Thus, in summary our results support the concept that the maneuver of over-expression of 4F2hc results in the formation of 4F2hc monomers and homodimers, which are responsible for cell transformation.

**Impact of 4F2hc depletion in tumorigenesis**

Three strategies to deplete 4F2hc expression in cells were implemented. In the first instance, miRNA were developed to deplete 4F2hc expression. Two out of five miRNA designed depleted 4F2hc to less than 10% in transient transfection in HeLa cells. Then, these miRNA and a negative control were introduced in the lentivirus vector pWPI together with GFP in HeLa and human colon adenocarcinoma cells. Depletion of 4F2hc expression reduced tumorigenicity by increasing the latency phase by impairment of implantation/initial proliferation of tumor cells in nude mice, but without decreasing tumor growth thereafter. 4F2hc-silenced HeLa cells showed a reduced mitogenic response to serum or phorbol esters, reduced Akt and ERK1/2 activation in response to laminin and fibronectin, and presented lower levels of secreted active MMP-2 together with an increase in galectin-3 (Santiago-Gómez et al., in preparation). All these effects strongly support the role of 4F2hc in tumorigenicity. Unfortunately, HeLa and colon adenocarcinoma cells selected for GFP expression by cell-sorter (i.e., positive for miRNA expression) rapidly lost the population of positive cells. The recovery of non-transduced cells precluded the use of this strategy for most of our experiments.

Secondly, in collaboration with Prof. Mark H Ginsberg, we used CD98hc-null MEFs, which were generated by infecting CD98hc-conditional knockout MEFs
with adeno-CRE encoding CRE recombinase (Feral et al., 2007). These cells were transduced with human 4F2hc mutant Δ86 (deletion of the N-terminal first 86 residues) or with the empty lentivirus vector pWPI. Only 2 cell-passages, after sorting cells for the lack of expression of endogenous mouse 4F2hc and maintaining the cells without β-mercaptoethanol in the medium, were enough to recover ~70% and ~90% of cells expressing endogenous mouse 4F2hc, respectively. Growing the cells in β-mercaptoethanol or N-acetylcysteine delayed the recovery of cells expressing endogenous mouse 4F2hc (~50% of cells expressing endogenous mouse 4F2hc in the group transduced with the empty vector). Thus, reducing agents or substrate supply for glutathione synthesis increases the cell growth of 4F2hc knockout MEF cells (González, de la Ballina, Palacín; unpublished results). A similar phenotype was reported for the null knockout of the 4F2hc-light subunit xCT (the x_c^- glutamate/cystine exchanger) in MEFs (Sato et al., 2005).

Lastly, in collaboration with Dr Chloe Feral, we used fibroblasts derived from 4F2hc null knockout mouse ES cells and transformed with SV40 (Feral et al., 2005). This is the only stable cell system with no recovery of endogenous expression of 4F2hc available. Similar wild-type fibroblasts express three 4F2hc-associated light subunits (LAT1, y^+LAT2 and xCT) and the corresponding amino acid transport activities (systems L, y^+L and x_c^-). As expected the knockout cells are devoid of these transport activities (de la Ballina, Feral, Palacín; unpublished results). Different mutants of human 4F2hc are currently transduced in these 4F2hc knockout cells to study amino acid transport function, β-integrin signaling, cell spreading and tumor formation.

**Interaction of 4F2hc with galectin-3**

All the screenings performed looking for glycans or proteins interacting with the ectodomain of 4F2hc (4F2hc-ED) revealed no candidate ligands. In these experiments N-glycosylated (produced in HEK293 cells) or non-glycosylated (produced in E. coli) 4F2hc-ED were used as a bait to search for interaction in
glycan-chips (Consortium for Functional Glycomics-USA) or solubilized plasma membranes from different cells (HeLa, CHO, Jurkat). No significant interaction with any of >200 different glycans was obtained, and mass spectroscopy analysis of 4F2hc-ED-affinity purified proteins was inconsistent to indentify protein ligands. Even so, it is worth mentioning that in several cell types (transfected and non-transfected HeLa cells, oocytes injected with human 4F2hc mRNA and in transfected CHO cells) a complex of ~200 kDa of 4F2hc, which do not correspond to 4F2hc homodimers (~160 kDa), was detected. DTT reduced this complex yielding the 4F2hc monomer. A disulphide bridge involving residue Cys330 (located in the ectodomain of 4F2hc) stabilizes the 200 kDa complex (de la Ballina, Palacín; unpublished results). TAP affinity purification of 4F2hc is currently in progress to analyze the nature of the complex by mass spectroscopy. The human 4F2hc mutant Cys330Ser has a dominant negative effect on the fusion of cells induced by specific 4F2hc antibodies (Okamoto et al., 1997). Thus, the Cys330-dependent 200 kDa 4F2hc complex might be a relevant component for the multiple functions of 4F2hc beside amino acid transport.

Galectin-3 has been revealed as a ligand of 4F2hc (Dong and Hughes, 1997). Indeed, it has been suggested that 4F2hc-galectin-3 interaction has a role in activation of macrophages and placental syncytium formation (Dalton et al., 2007; MacKinnon et al., 2008). We analyzed the physical interaction of galectin-3 and 4F2hc-ED by surface Plasmon resonance. This interaction requires N-glycosylation of 4F2hc-ED at any of the four N-glycosylation sites of the protein. Thus, a non-glycosylated mutant of 4F2hc-ED does not interact with galectin-3 (Fort, Palacín; unpublished results).

**Role of the ectodomain of 4F2hc in tumorigenesis**

This part of the project is still in progress due to the difficulties in establishing a cell system devoid of 4F2hc. ES cells knockout for 4F2hc and fibroblast derived from these cells have been finally selected to analyze the role of the ectodomain of 4F2hc. Three types of experiments are in course:
i) transduction of several chimeras of 4F2hc and CD69 implying the ectodomain of 4F2hc and including mutant Cys330Ser of human 4F2hc to study the formation of teratocarcinomas in nude mice, ii) transduction of N-glycosylated and non-glycosylated forms of human 4F2hc to study the role of galectin-3 in β-integrin signaling, and iii) transduction of several mutants implying relevant structural residues in the ectodomain of 4F2hc to study their impact on amino acid transport, β-integrin signaling, and tumorigenesis.

References


3. Relevance of the results

This project explored the role of the ectodomain of 4F2hc in the ability of this protein to transform cell and induce tumor formation. The project had a basic science character with no immediate translation to clinics. The following are the most relevant results at present:

1) The atomic structure of the ectodomain of 4F2hc and its dimer was solved at 2.1 Å.
2) Heterodimers of 4F2hc are over-expressed in several tumor tissues, including colorectal cancer. In tumor cell lines the expression of 4F2hc with different degree of glycosylation was substantiated.
3) Over-expression of 4F2hc in different cell systems results in the formation of 4F2hc homodimers. This homodimers are stabilized by a disulphide bridge between Cys109 residues. These homodimers might be at the basis of the cell transformation induced by 4F2hc.
4) 4F2hc silencing results in a higher latent period in the growth of tumors produced by subcutaneous injection of HeLa and human colon adenocarcinoma cells in nude mice.
5) 4F2hc knockdown HeLa cells also showed lower mitogenic response, integrin signaling and metalloproteinase MMP-2 expression and activity.
6) Galectin-3 binds to the ectodomain of 4F2hc. This interaction requires at least one of the four N-glycosylation sites of 4F2hc.
7) 4F2hc forms a 200 kDa complex with a Cys330-intervening disulphide bridge in different cell types. The nature of this complex is, to our knowledge, unknown.

4. Publications
