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Technical note

Improvement of immunodetection of the transcription factor C/EBP homologous protein by western blot

Jonathan Turpin[1a], Etienne Frumence[1a], Daed El Safadi[1], Olivier Meilhac[2,3], Pascale Krejbich-Trotot[1]* and Wildriss Viranaïcken[1] *

[1]Université de La Réunion, INSERM UMR 1187, CNRS 9192, IRD 249, Processus Infectieux en Milieu Insulaire Tropical (PIMIT), Plateforme CYROI, 2, rue Maxime Rivière, 97490 Sainte-Clotilde, Ile de La Réunion. France.

[2]Université de La Réunion, INSERM UMR 1188, Diabète athérothrombose Thérapies Réunion Océan Indien (DéTROI), Plateforme CYROI, 2, rue Maxime Rivière, 97490 Sainte-Clotilde, Ile de La Réunion. France.

[3]CHU de La Réunion, Saint-Denis, France.

[a]Contribute equally to this work

*Correspondence: wildriss.viranaicken@univ-reunion.fr (W.V.); pascale.krejbich@univ-reunion.fr (P.K.T.); Tel.: +33-262938829

Abstract

Accumulation of misfolded proteins within the endoplasmic reticulum (ER) induces an unfolded protein response (UPR) that either restores homeostasis or triggers apoptosis in case of adaptation failure. The three activated branches of UPR lead to IRE1-, PERK- and ATF6- dependent transcriptional induction of the gene encoding the transcription factor C/EBP homologous protein (CHOP) which plays an important role in apoptosis induction. In conventional immunoblotting conditions, detection of CHOP is a difficult task. Using a fixation step, we have optimized the detection of CHOP and this method provides a valuable tool to decipher CHOP involvement in UPR.

Running title: Sensitive western blotting detection of CHOP

Keywords: CHOP; Unfolded Protein Response; Western blot; Immunodetection; Nitrocellulose.

Introduction

Endoplasmic reticulum (ER) is involved in many cellular processes such as lipid synthesis, cellular calcium homeostasis, protein synthesis and folding. Proteostasis is maintained by the ER through

many biological processes that control and participate in the correct folding of newly synthesized proteins. In some conditions the ER can be submerged by overexpressed, unfolded or misfolded proteins. To overcome this stress, the cell engages the unfolded protein response (UPR) through three activated pathways, namely ATF6, IRE-1 and PERK. Upon ER stress, activated ATF6 will be translocated to the Golgi apparatus, cleaved and then reach the nucleus to drive transcription upregulation of genes encoding chaperone proteins like GRP78/94 [1]. The IRE-1 pathway will lead to the activation of the Transcription Factor XBP1 through an endonuclease spliced Xbp1 mRNA (Xbp1s). This branch of the UPR will be mainly devoted to ER-associated protein degradation (ERAD) [2]. Lastly, the PERK pathway will be quickly activated to limit protein translation with phosphorylation of the initiation factor eIF2α. However, this phosphorylation allows a selective translation of specific mRNA including the one coding for ATF4 which is a transcriptional factor that upregulates CHOP expression. CHOP, alternatively named GADD153 or DDIT3 is a factor directly involved in ER-mediated apoptosis through the regulation of genes like PUMA, BIM and Bcl2 [3]. CHOP is therefore an essential marker for the monitoring of the UPR and an indicator of ER stress resolution and cell fate.

To monitor specific cell response markers, Western blot is a commonly used method allowing semi-quantification of target proteins after migration and separation of cell extracts on acrylamide gel and their transfer on nitrocellulose (NC) or PVDF membrane. Western blot sometimes leads to very disappointing results with no signal from the target protein. This lack of signal despite a great amount of proteins could be linked to antibody recognition, loss of a conformational epitope, transfer efficiency or loss of proteins during the washing and incubation steps [4]. To improve the signal, many protocols exist, but none of them solve the problem for all proteins. As detection of CHOP by Western blot remain a challenge for all research work that aims to characterize stress [5], here we have improved a classic blotting protocol to follow CHOP protein level.

2. Materials and methods

2.1. Cell line, antibody and reagent

A549-Dual™ cells (InvivoGen, Toulouse, France, a549d-nfis) designated hereafter as A549 cells were maintained in MEM medium supplemented with 10% heat-inactivated FBS. A549 cells were maintained in growth medium supplemented with 10 $\mu g.mL^{-1}$ blasticidin and 100 $\mu g.mL^{-1}$ zeocin (InvivoGen, Toulouse, France). CHOP antibody (D46F1 Rabbit mAb #5554) was from cell Signalling Technology (Ozyme, Saint-Cyr-l'École, France). Glutaraldehyde (GA) and Thapsigargin (TG) were purchased from Sigma-aldrich (Humeau, La Chapelle-Sur-Erdre, France). The nitrocellulose membrane, amersham Protran Premium 0.22 or 0,45 µm were from GE HEALTHCARE (GE, Buc, France).

2.2. Western blot

Cells (5.10^6) were lysed in 0.5 mL of radioimmunoprecipitation assay buffer RIPA buffer (Sigma-aldrich Humeau, La Chapelle-Sur-Erdre, France) at 4°C for 10 min. Lysate were sonicated at 0°C for 10 S of pulse at 80% at 0.5 cycle using a Mixsonic sonicator. Proteins from total cell extract was quantified using BCA reagent according to manufactured instructions (Sigma-aldrich Humeau, La Chapelle-Sur-Erdre, France). Separation of total cell extract by SDS-PAGE and transfer to nitrocellulose were performed as previously described [6]. Briefly proteins extracts were treated with Laemmli buffer and heat at 95°C for 5 min. 10 or 20 µg of proteins were separated on precast NuPAGE gels 4-12% (Thermofischer, France) or in house SDS-PAGE at 10% with 37.5/1 ratio of acrylamide/bisacrylamide as describe previously [7]. The standard transfer was performed, as initially describe [8], on NC Protan supported membrane with a 0.45 µm pores (Amersham, GE, Buc, France). After transfer, membranes were rapidly washed with Phosphate Buffered Saline (PBS) and fixed with glutaraldehyde at several different concentrations during indicated times. After 3 wash steps with PBS containing Tween 20 at 0.1% (v/v) (PBS-T), the membrane was saturated using 5% non-fat dry milk in PBS-T. Primary antibodies were used at 1:1000 dilutions ($1 \mu g.mL^{-1}$) in PBS-T. Anti-rabbit immunoglobulin-horseradish peroxidase and anti-mouse immunoglobulin-horseradish peroxidase conjugates from Abcam (Cambridge, UK) were used as secondary antibody according to manufacturer's instructions (1:2000 in PBS-T, 0.5 $\mu g.mL^{-1}$). Blots were revealed with enhanced chemiluminescence, ECL prime detection reagents (GE, Buc, France) using an Amersham Imager 680 (GE, Buc, France).

2.3. Immunofluorescence Assay

A549 cells grown on glass coverslips were fixed with 3.7% formaldehyde at room temperature for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 4 min. Coverslips were incubated with primary antibodies (1:1000 dilution, $1 \mu g.mL^{-1}$) in 1% BSA-PBS. Antigen-antibody binding was visualized with Alexa 594 Fluor-conjugated secondary antibodies (1:1000, Invitrogen). Nucleus morphology was revealed by 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining. The coverslips were mounted with VECTASHIELD® (Clinisciences, Nanterre, France) and fluorescence was observed using a Nikon Eclipse E2000-U microscope. Images were captured and processed using a Hamamatsu ORCA2 ER camera and the imaging software NIS-Element AR (Nikon, Tokyo, Japan).

3. Results

3.1. CHOP is detected by immunofluorescence assay but not by conventional western blot technique.

In order to follow CHOP induction upon ER stress in A549 cells and to validate the use of CHOP antibody, CHOP intracellular imaging was performed by immunodetection and analysis by fluorescence microscopy. A549 cells were grown on coverslips and stimulated for 4 hours by 1µM of thapsigargin (TG), an inhibitor of SERCA leading to intracellular Ca^{2+} release and ER stress. CHOP was not detectable in non-treated cells (Control) but we evidenced a strong nuclear signal

in A549 TG-treated cells (Figure 1). This result shows that our antibody recognizes CHOP and that CHOP is expressed and localized within the nucleus of A549 cells upon ER stress. To assess CHOP protein levels in A549 cells, we used a total cell extract for western blot. No signal could be observed following a classical immunoblot protocol, even when a more sensitive, enhanced chemiluminescent substrate was used (ECL select from GE, data not shown). Different conditions were tested including proteins preparation with different lysis buffers (RIPA, Urea lysis buffer, Laemmli buffer), different ratio of acrylamide/bisAcrylamide for the SDS-PAGE to increase transfer to membrane (ie 37.5/1 or 74/1) or commercially available precast gels (NuPage). Transfer parameters were also tested (transfer time 30 min to 16h, transfer at room temperature (25°C) or at 4°C, transfer buffer with methanol, ethanol or isopropanol, semi-dry transfer or liquid transfer, transfer on PVDF membrane or on NC membrane (Protan with 0.2 µm pores). As it has been previously shown that milk can cause a release of proteins from the transfer membrane during the saturation stage [9], we also tested the alternative saturation with 1%BSA during several times. In all cases, CHOP could not be detected by western blot in our conditions even by the use of different antibodies against CHOP from other suppliers (data not shown). Therefore, CHOP detection by classical western blot techniques was unsuccessful.

3.2. Membrane fixation improved CHOP signal for western blot.

As western blot is a gold standard technique to evaluate protein expression modulation and their relative abundance in samples, we tried to optimized CHOP detection by this method. As immunofluorescence allowed a validation of our antibody, with the expected detection of CHOP nuclear localization upon UPR (Figure 1), we aimed at solving the lack of detection by WB. It should be noted that, according to the supplier, the antibody used was supposed to be suitable for both immunofluorescence and Western blot. Previous studies have already shown that one major issue of this technique was the loss of proteins from the blotted membrane during incubation and washing steps, especially for small acidic proteins like caspase-3 or insulin (Table 1) [10,11]. Using the ProtParam tool (https://web.expasy.org/protparam/), we found that CHOP is a small protein more acidic than caspase-3 or insulin (Table 1). We thus tested whether our difficulties to detect this protein could be related to a poor retention of CHOP to the NC membrane.

Using glutaraldehyde, we tested chemical protein fixation on the membrane before blot saturation and immunolabeling steps. Glutaraldehyde (GA) is a fixative agent forming crosslinks between macromolecules. It is generally used during sample preparation for immunohistochemistry or electron microscopy. Several articles have described membrane fixation [10,12,13], but this technique does not work for all proteins, as conformational changes may occur within the recognized epitopes of the protein of interest. Without available data on our target protein CHOP, we tested this pre-fixation method. NC membrane were incubated after the gel transfer with 0.5% (V/V) glutaraldehyde in PBS for 20 min, and washed three times with PBS-T. Immunolabelling was then performed as usual (see material and methods).

Without GA pre fixation of the blot, three main non-specific bands around 70, 60 and 30 kDa were observed after immunodetection of CHOP. None of these signals match with CHOP, which is expected at 26 kDa as apparent molecular weight (Figure 2A). Following treatment with GA 0.5% for 10 min, the expected 26 kDa signal appeared (black arrowhead) and was specific of cells treated with the UPR inducer (Figure 2A). Moreover, we observed a general background decrease and less signal related to non-specific bands. Finally, GA enhanced retention of CHOP on the NC and improved antibody interaction specificity by eliminating non-specific interactions.

The fixation condition could be improved using different incubation times or GA concentrations. To determine the optimal concentration, GA at 0.25, 0.5, 1, and 2% were tested for 10 min for protein fixation on NC membranes. Glutaraldehyde at 0.5% was found to be the optimal concentration for CHOP immunodetection (Figure 2B).

For incubation time optimization, NC membranes were incubated for 5, 10 or 20 min with GA at 0.5% in PBS. The optimal fixation time for detecting CHOP was 5 min (Figure 2C). We can conclude that protein fixation with 0.5% glutaraldehyde for 5 min improved CHOP and allowed its detection after western blotting. This optimization of the most obvious way to detect a protein level gives access to an important information on this specific indicator of ER stress and UPR.

4. Conclusion.

ER stress is a common cellular response involved in conformational diseases, metabolic syndrome and viral infections [14][10]. GADD153/CHOP is one of the main actors of ER stress resolution that seals the cell's fate in terms of survival or death and its detection by a gold standard method like western blot is indispensable. Here we have improved CHOP immunodetection by western blot with a glutaraldehyde fixation step, providing a great tool to analyze ER stress status in cell extract samples.

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Figure Legends

Table 1. Comparison of several biochemical characteristics of the CHOP, insulin and caspase-3 proteins.

Figure 1. CHOP detection by immunofluorescence during UPR activation. A549 cells were grown on coverslips and treated with TG for 4h. Cells were fixed with 3.7% PFA and CHOP was detected by immunofluorescence with DAPI counterstaining of nuclei.

Figure 2. Improvement of CHOP detection by western blot. (**A**) Total A549 cell extracts treated or not with TG for 4h were separated by SDS-PAGE followed by coomassie-blue (C.B) staining or followed by transfer to NC membrane for immunoblotting. After transfer, the membranes were fixed for 10 min with 0.5% glutaraldehyde (GA) in PBS (**A**) or 10 min with different concentrations of GA in PBS (**B**) or for the indicated time with 0.5% of GA in PBS (**C**). The black arrowhead indicates CHOP signal. In (B) for the loading control, the upper part of the membrane (>38 kDa) was incubated with α-tubulin without any GA fixation.

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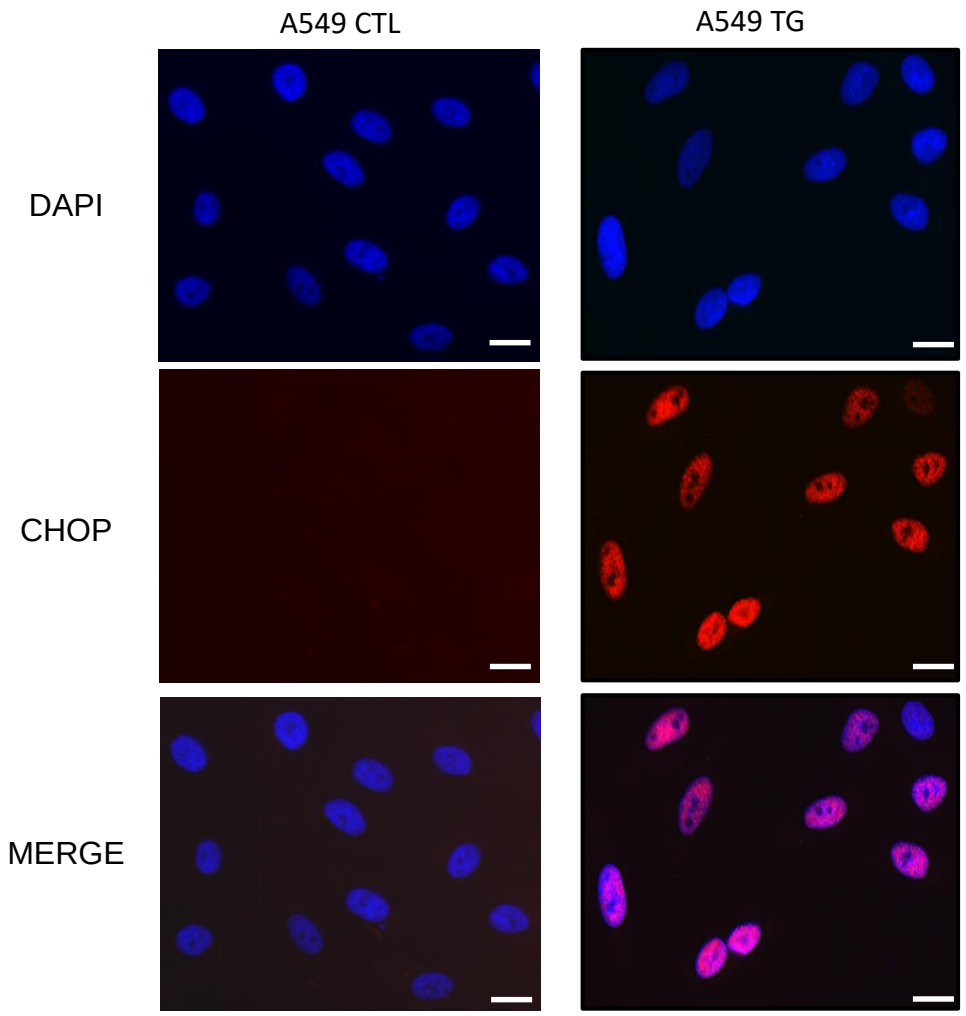
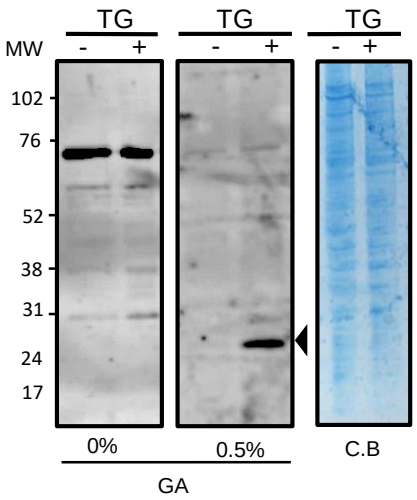
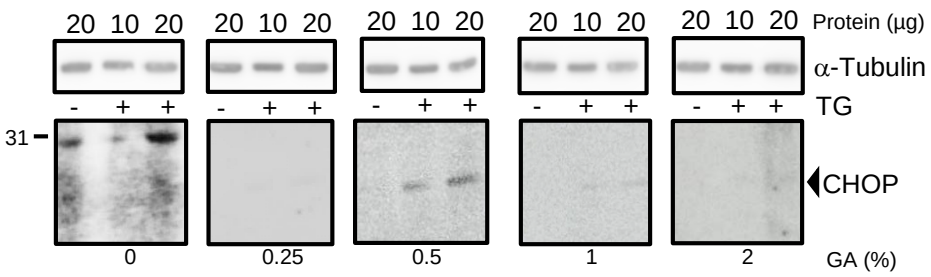


Figure 1

A


TG
TG
TG
MW
- +
- +
- +
102
76
52
38
31
24
17
0%
0.5%
C.B
GA

B


20 10 20
20 10 20
20 10 20
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20 10 20
Protein (μg)
α-Tubulin
- + +
- + +
- + +
- + +
- + +
TG
31
CHOP
0
0.25
0.5
1
2
GA (%)

C

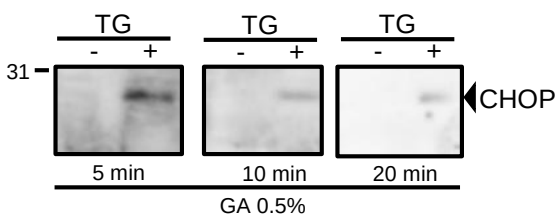
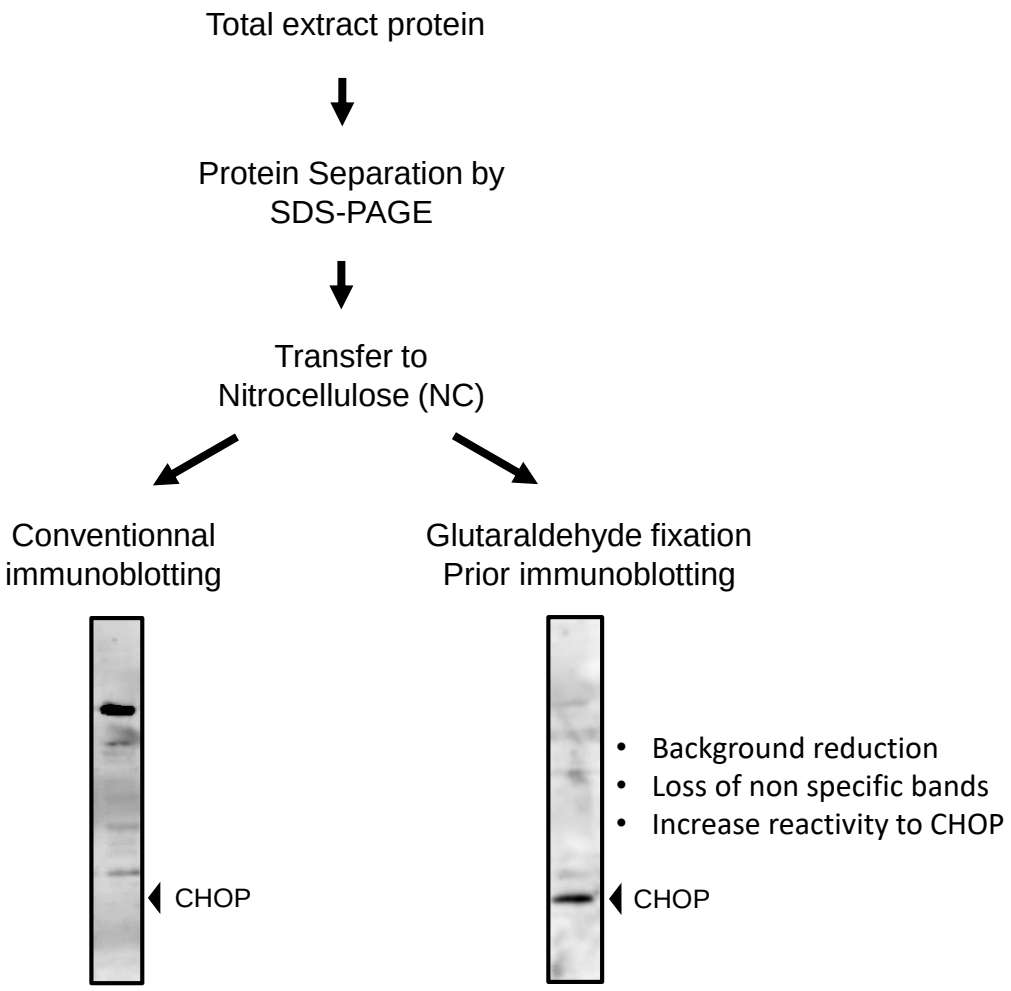

TG
TG
TG
- +
- +
- +
31
CHOP
5 min
10 min
20 min
GA 0.5%

Figure 2

	CHOP	CASP-3	INSULIN
UnitProt number	P35638	P42574	P01308
Number of amino acids	169	277	110
Molecular weight (kDa)	19.174	31.607	11.980
Theoretical pI	4.61	6.09	5.22

Table 1



Graphical abstract