SNARE expression and distribution during 3T3-L1 adipocyte differentiation

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Abstract Differentiation of 3T3-L1 cells into adipocytes presupposes the expression of the glucose transporter isoform GLUT4 and the acquisition of insulin-dependent GLUT4 translocation from intracellular storage vesicles to plasma membrane. This ability to translocate GLUT4 depends on the presence of a set of proteins of the SNARE category that are essential in the fusion step. The expression and levels of some of these SNARE proteins are altered during 3T3-L1 differentiation. Levels of the v-SNARE protein cellubrevin and of the t-SNARE protein syntaxin 4 were increased in this process in parallel to GLUT4. However, the levels of SNAP-23, another t-SNARE, were maintained during differentiation. Immunofluorescence images of SNAP-23 showed the initial distribution of this protein in a perinuclear region before differentiation and its redistribution towards plasma membrane in the adipocyte form. These results suggest a capital role in the expression levels and cellular distribution, during 3T3-L1 differentiation, of SNARE proteins involved in the late steps of GLUT4 translocation.

Key words: GLUT4 translocation; 3T3-L1 cell line; SNARE protein

1. Introduction

The translocation of the glucose transporter GLUT4 to the cell surface is the main mechanism for insulin-dependent glucose uptake in muscle fibers and adipocytes. This event is mediated by the fusion of GLUT4-containing intracellular vesicles (GLUT4 storage vesicles, recycling endosomes) with the plasma membrane [1–5]. The fidelity of this mechanism relies on, at least in part, a specific set of SNARE proteins [6,7] that are essential in the fusion step [8]. The SNARE proteins involved are synaptobrevin 2 and cellubrevin as v-SNARE, which are localized in intracellular membrane compartments, and syntaxin 4 and SNAP-23 as t-SNARE [9–18] at the plasma membrane. The importance of these proteins was evidenced by the use of clostridial toxins that selectively cleaved both synaptobrevin 2 and cellubrevin, blocking the insulin-dependent GLUT4 translocation [11,19–22], and recombinant forms of these SNARE proteins, which also interfered with the insulin-dependent GLUT4 translocation [11,13–15,18,23,24].

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The 3T3-L1 preadipocyte cell line has been widely used as an 'in vitro' model to study the insulin-dependent GLUT4 translocation. This cell line is a substrain of the 3T3 fibroblast cell line developed through clonal isolation [25]. These cells undergo a conversion from preadipose to adipose-like cells under confluent conditions in the presence of dexamethasone, isobutylmethylxanthine and insulin [26–28]. This conversion confers to 3T3-L1 cells the ability to newly synthesize GLUT4 [29,30] and translocate it from intracellular vesicles to the plasma membrane under insulin stimulation [29,31–33]. Concomitantly, an increase in the relative expression of cellubrevin [9], in parallel to GLUT4, was also found in 3T3-L1 adipocytes suggesting that cellubrevin accompanies the movement of GLUT4 found in the regulated endosomal pathway in response to insulin [9]. In contrast SNAP-23 levels were unaffected in 3T3-L1 cells during differentiation [34], and there are still no compelling data for VAMP2 and syntaxin 4.

Changes in v- or t-SNARE protein expression may indicate additional requirement for an existing function or acquisition of new functional roles after differentiation. In any case, the cognate v- or t-SNARE should follow similar differences in expression or, at least, undergo a redistribution of the existing protein to afford new or additional functional requirements. The aim of the present work is to explore possible differences in t-SNARE protein expression or distribution comparing 3T3-L1 cells before and after differentiation. As results, we show an increase in syntaxin 4 expression in differentiated 3T3-L1 adipocytes, in addition to the previously shown expression of cellubrevin [9] and the redistribution of SNAP-23 in differentiated 3T3-L1 adipocytes. With these findings, it is suggested that the appearance of GLUT4 in the process of 3T3-L1 differentiation is parallel to differences in expression or redistribution of SNARE proteins directly involved in the insulin translocation of the glucose transporter.

2. Materials and methods

2.1. Antibodies

Cytosolic fragments of syntaxin 2, 3 and 4 were obtained by reverse transcription-PCR from rat liver. *Bam*HI and *Eco*RI restriction sites were introduced in forward and backward primers of all syntaxin isoforms and cellubrevin, to facilitate subcloning of the amplified DNA sequences into p-GEX 4T-1 GST fusion protein expression vector (Pharmacia). Each purified protein was injected in two rabbits and the immune sera were obtained by standard procedures. To avoid cross-reaction of the sera between the different syntaxin isoforms, each serum was absorbed with beads bound to the GST–syntaxin isoforms except for the one it was generated against. The polyclonal antibody that recognizes GLUT4 [35] was raised against the 15C-terminal peptide from GLUT4. The rest of the antibodies were ob-

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tained from different companies; Golgi 58K protein monoclonal antibody (Sigma, clone 58K-9), syntaxin 1 monoclonal antibody (Sigma, clone HPC-1), Munc-18 monoclonal antibody (Transduction Laboratories), α-tubulin monoclonal antibody (Sigma, clone DM1A), anti-rabbit Na/K-ATPase β_1 subunit monoclonal antibody (Upstate Biotechnology). The antibody against SNAP-23 and cellubrevin were obtained as described [36,37].

2.2. Cell culture

3T3-L1 cell line was obtained from the American Type Culture Collection (CL-173). All the experiments were made between passages 4 and 12. The cells were grown in Dulbecco's modified Eagle's medium (Gibco) containing high glucose, L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg of streptomycin (Sigma) in a humidified incubator with 5% CO_2. Cells were differentiated into adipocyte phenotype as described by [38].

2.3. Immunocytochemistry

The cells on cover slips, previously treated with polylysine (Sigma), were fixed with 4% paraformaldehyde for 20 min and washed three times with phosphate-buffered saline (PBS). They were first incubated for 1 h at room temperature with the blocking solution (PBS, Triton X-100 0.2%, gelatin 0.2% and FBS 10%) and then with the primary antibodies in the same buffer (but only with 1% of FBS) overnight at 4°C. The primary antibodies were used at the following dilutions: anti-cellubrevin 1:50, anti-syntaxin 4 1:50, anti-SNAP-23 1:50 and anti-Golgi 58K protein 1:200. After incubation with primary antibodies, cells were washed three times with PBS with 0.2% Triton X-100 at room temperature for 10 min each. FITC-conjugated anti-rabbit immunoglobulins or Texas Red-conjugated anti-mouse immunoglobulin secondary antibodies (Amersham) were used at 1:200 dilution for 1 h at room temperature. Cells were washed three times with PBS containing 0.2% Triton X-100 at room temperature for 10 min and then the cover slips were mounted with fluorescent mounting medium (Dako). Slides were observed in a Leica TCS 4D confocal microscope (Serveis Científico-Tècnics, Universitat de Barcelona, Barcelona, Spain).

2.4. Immunoblots

For each experimental condition, 10 μg of total protein was loaded per lane. After SDS–PAGE, the proteins were transferred onto nitrocellulose membranes (Bio-Rad). After blocking using 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20, membranes were incubated overnight at 4°C with the primary antibodies in the same buffer. Unless otherwise specified, the primary antibodies were used at the following dilutions: anti-syntaxin 2 1:500, anti-syntaxin 4 1:500, anti-cellubrevin 1:500, anti-SNAP-23 1:500, anti-Munc-18 1:1000, anti-GLUT4 1:800, anti-rabbit Na^+/K^+-ATPase 1:1000 and anti-α-tubulin 1:3000. After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase, the blots were developed using the enhanced chemiluminescence method.

For silver-stained gels, proteins were resolved by SDS–PAGE (7.5 μg of total protein per lane) and developed as described [39].

3. Results

3.1. Protein expression

3T3-L1 cells undergo biochemical and morphological changes upon differentiation. Changes in the protein levels can be evidenced by silver staining of cell extracts resolved by SDS–PAGE (Fig. 1). The variations in protein composition might include modifications in the levels or cellular distribution of those proteins involved in newly acquired cellular mechanisms, i.e. GLUT4 translocation. Some bands with increased staining after differentiation may correspond to proteins previously described (i.e. GLUT4, Fig. 1). However it is very difficult to recognize proteins only from silver-stained gels. Thus, we compared the levels of those proteins involved in insulin-dependent GLUT4 translocation in differentiated and non-differentiated 3T3-L1 cells by Western blot. Similarly to Fig. 1, three situations in the differentiation process were chosen: cells in the initial non-differentiated fibroblastic phenotype; confluent cells in differentiating medium for 2 days; and full-differentiated adipocytes (see Section 2).

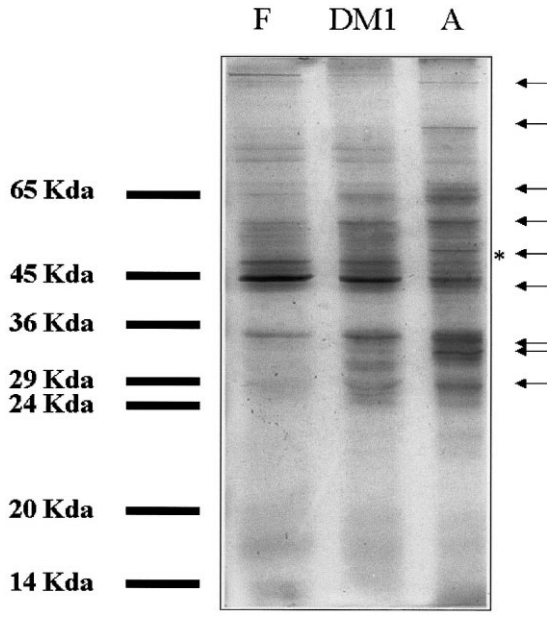

Fig. 1. Silver staining of different protein patterns in the process of differentiation in 3T3-L1 cell line. F: Homogenate of 3T3-L1 fibroblasts (preadipose cells), DM1: 3T3-L1 cells in differentiating medium 1 (2 days after confluence with insulin, dexamethasone and isobutylmethylxanthine, as specified in Section 2) and A: differentiated 3T3-L1 adipocytes. Arrows indicate bands with a new or increased expression in adipocytes; an asterisk indicates a band that, according to its molecular weight, could correspond to GLUT4.

Initially, in an attempt to characterize the expression and distribution of t-SNARE in the plasma membrane and the efficiency of the antibodies used in this work, the presence of some t-SNARE proteins that could be involved in traffic of intracellular membrane compartments with plasma membrane was checked.

Due to the high degree of homology between syntaxin isoforms and to eliminate possible cross-reactions, anti-syntaxin 2, 3 and 4 antisera were first incubated with recombinant syntaxins (1–4) except for the specific form they were produced against. The absorbed sera (see Section 2) were checked for specificity using recombinant cytosolic fragments of syntaxin isoforms and Western blot analysis. In all cases no cross-reactivity was observed (Fig. 2A).

As previously described in adipocytes and 3T3-L1 cells [9,10,17,40,41], syntaxin 2 and 4, cellubrevin and SNAP-23 were found in differentiated 3T3-L1 adipocytes, while SNAP-25, syntaxin 1 and syntaxin 3 were not consistently found by Western blot of differentiated 3T3-L1 cell extracts (data not shown).

In accordance with changes in the protein expression observed on silver-stained SDS–PAGE from cell extracts, Western blot analysis also showed differences in the relative amounts of several identified proteins comparing the fibroblastic and the adipocyte 3T3-L1 phenotypes. In particular, and in addition to the previously shown changes in GLUT4 and cellubrevin expression [9], the relative levels of syntaxin 4 were also increased during differentiation as shown by West-

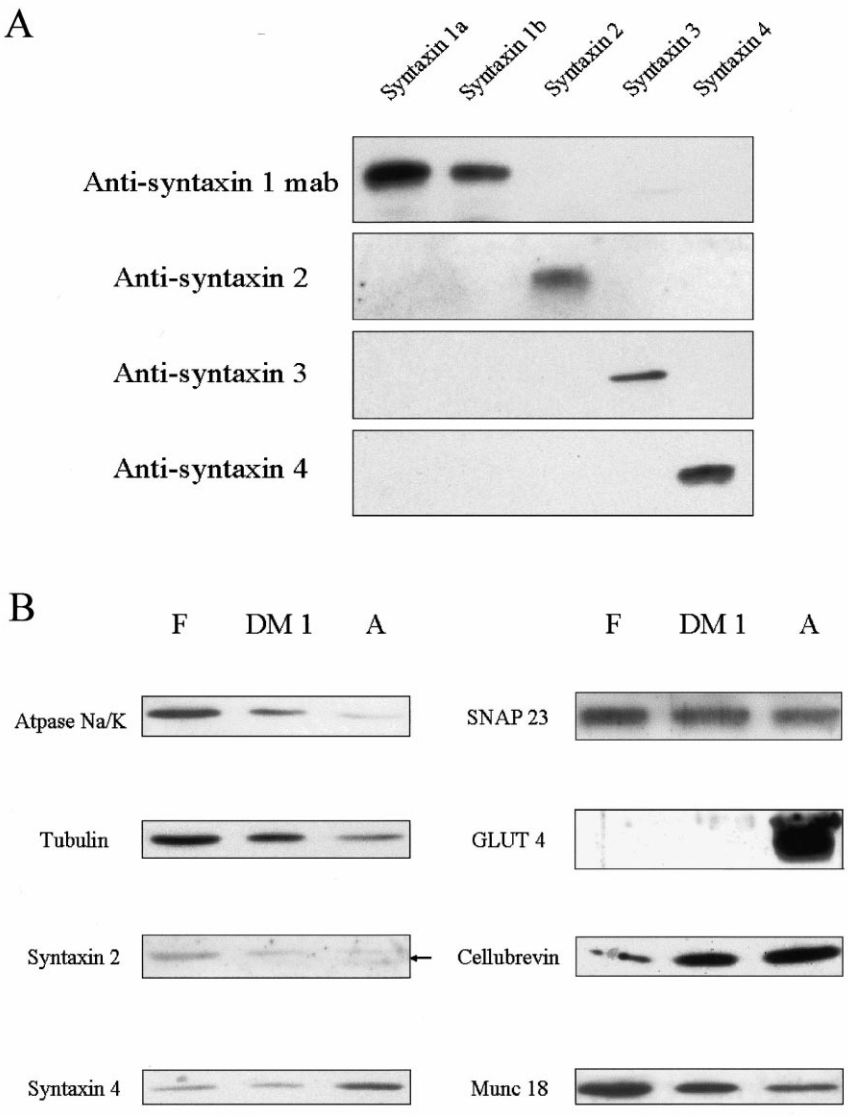

Fig. 2. Western blot analysis of SNARE proteins during the differentiation process in 3T3-L1 cell line. A: Western blot of different SNARE proteins in three steps of the differentiation process. F: Homogenate of 3T3-L1 fibroblasts (preadipose cells), DM1: 3T3-L1 in differentiating medium 1 (see Fig. 1) and A: 3T3-L1 mature adipocytes. Arrow indicates an additional band for syntaxin 2. B: Specificity of anti-syntaxin isoform sera. 10 ng of each recombinant syntaxin isoform (cytosolic fragment) per lane was loaded. Membranes were incubated with pre-absorbed sera (see Section 2) against each syntaxin isoform checking for cross-reactivity. The monoclonal antibody anti-syntaxin 1 (HPC-1) was used at 1:1000 dilution. Antisera against syntaxin isoforms were used at 1:100 dilution.

ern blot (Fig. 2B). Levels of SNAP-23, however, remained similar.

Finally, some other protein relative amounts were significantly decreased during differentiation, α-tubulin, Na^+/K^+-ATPase, Munc-18 and syntaxin 2 (Fig. 2B). In addition to decreased amounts of syntaxin 2, a second band of lower electrophoretic mobility appeared in differentiated 3T3-L1 cells (arrow, Fig. 2B).

3.2. Immunofluorescence studies

The distribution of SNAP-23, syntaxin 4 and cellubrevin was studied by immunofluorescence and confocal microscopy, in 3T3-L1 non-differentiated and differentiated cells. 3T3-L1 fibroblasts showed staining around the cell nucleus for all three proteins (Fig. 3A,C,E,G). Cellubrevin and syntaxin 4 displayed, in general, a more diffuse distribution, being more intense in the nuclear periphery for cellubrevin (Fig. 3A), while SNAP-23 had a more concentrated location, close to the cell nucleus (Fig. 3E,G). In 3T3-L1 adipocytes, cellubrevin was mainly located in a pole around the nucleus and in a diffuse pattern throughout the cytoplasm between lipid droplets (Fig. 3B). While some of the syntaxin 4 labeling was found in a diffuse intracellular compartment, its presence in plasma membrane was evident in full-differentiated 3T3-L1 adipocytes (Fig. 3D). Also in the adipose phenotype, SNAP-23 was still found in a structure surrounding the nucleus but also in the plasma membrane, clearly indicating a new location in the differentiated form of 3T3-L1 cells compared to the non-differentiated fibroblastic form (Fig. 3F,H).

Double staining immunofluorescence was performed in an attempt to define the intracellular localization of SNAP-23 around the nucleus. Golgi 58K protein antibody was used to check the co-localization of these proteins with SNAP-23. Fig. 4 shows a partial co-localization of these two proteins

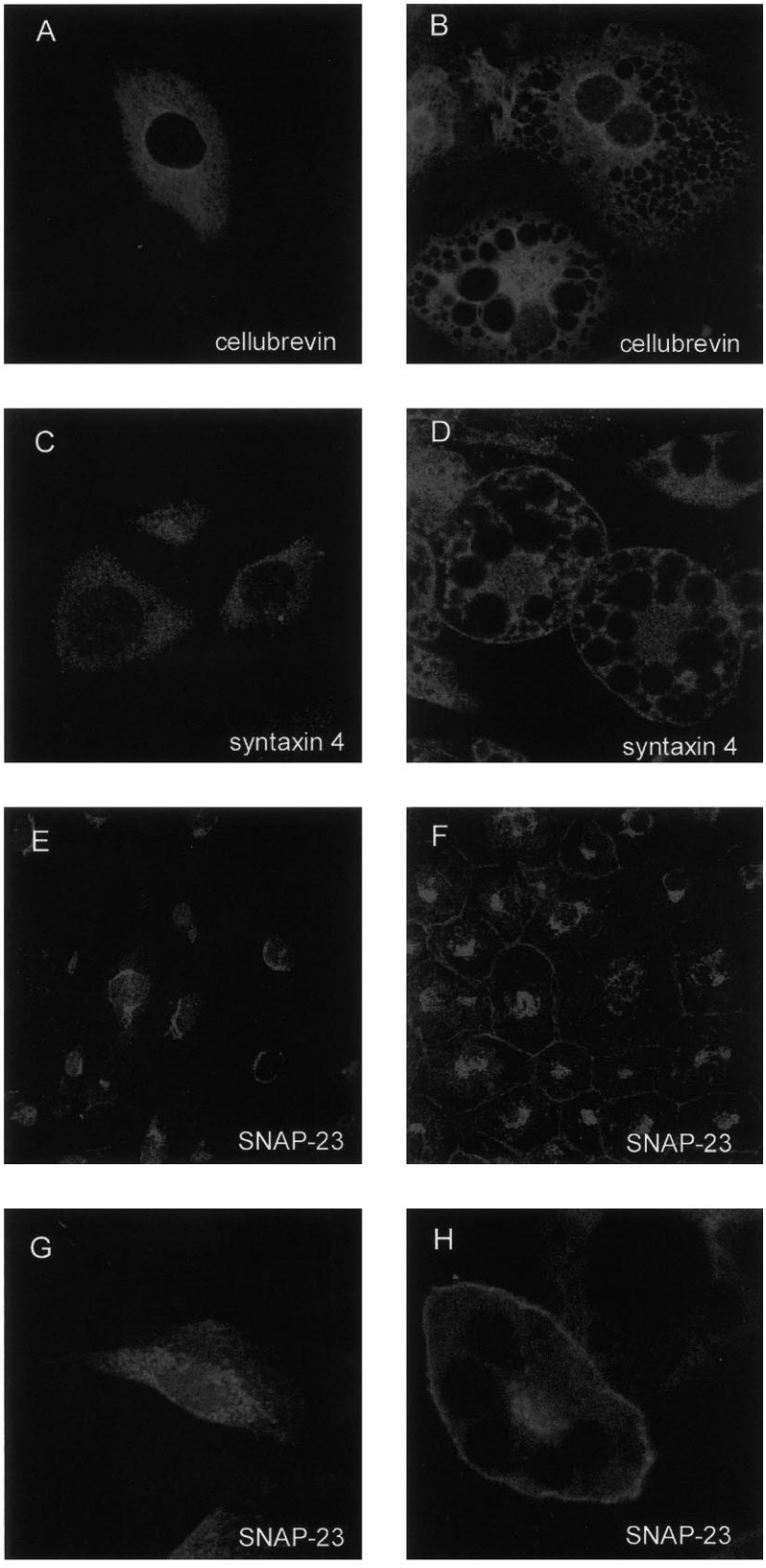
A
cellubrevin
B
cellubrevin
C
syntaxin 4
D
syntaxin 4
E
SNAP-23
F
SNAP-23
G
SNAP-23
H
SNAP-23
3T3-L1
non differentiated
3T3-L1
differentiated

Fig. 3. Immunolocalization of cellubrevin, syntaxin 4 and SNAP-23 in 3T3-L1 fibroblasts and adipocytes. Confocal microscopy was used to detect the presence of cellubrevin, syntaxin 4 and SNAP-23 in 3T3-L1 cells as described in Section 2. A, C, E and G show images of non-differentiated (fibroblasts) 3T3-L1 cells. B, D, F and H show images of 3T3-L1 differentiated adipocytes. A and B show the distribution of cellubrevin around the cell nucleus and cytoplasm in both phenotypes. C and D show syntaxin 4 clearly localized in the plasma membrane of the adipocytes and in a still not defined compartment in the cytoplasm of adipocytes and fibroblasts. E, F, G and H show different images of the distribution of SNAP-23 around the cell nucleus (probably Golgi complex) in 3T3-L1 fibroblasts (E and G) and 3T3-L1 adipocytes (F and H) at the plasma membrane only in the case of 3T3-L1 adipocytes.
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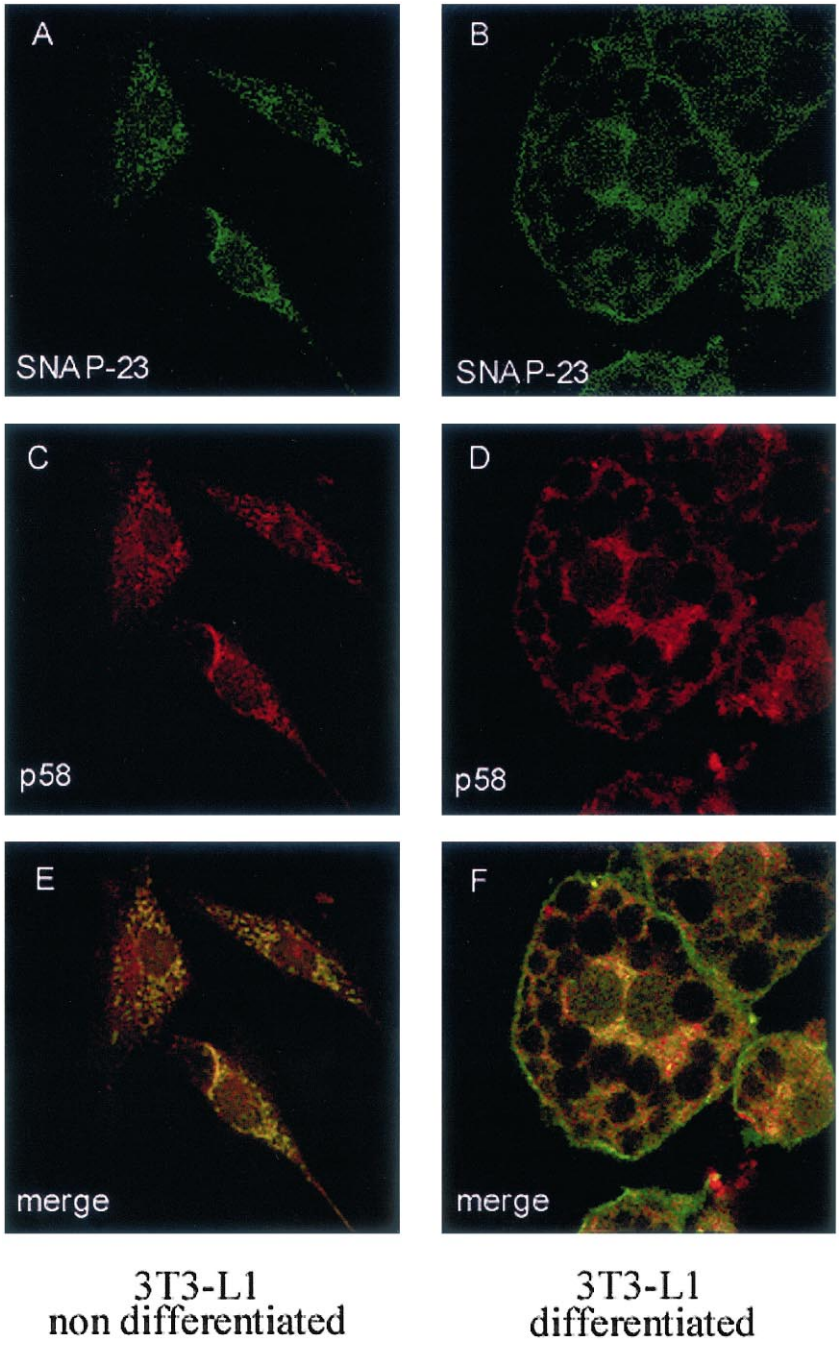

Fig. 4. Co-localization of SNAP-23 and the Golgi 58K protein in 3T3-L1 fibroblasts and adipocytes. Confocal microscopy and double immunofluorescence staining of SNAP-23 and Golgi 58K protein in 3T3-L1 cells were used to define SNAP-23 intracellular localization. A, C and E show staining of non-differentiated 3T3-L1 cells while B, D and F show staining of differentiated 3T3-L1 cells. A and B show distribution of SNAP-23 in both phenotypes, and C and D show Golgi 58K protein staining. E and F: Co-localization images. The double-immunostaining indicates that SNAP-23 partially co-localizes with the Golgi complex marker. Staining of SNAP-23 at the plasma membrane was found after 3T3-L1 cell differentiation into adipocyte (see also Fig. 3).

both in the non-differentiated and differentiated 3T3-L1 cells in the cytoplasmic area close to the cell nucleus, suggesting the presence of SNAP-23 in the Golgi complex both in 3T3-L1 fibroblasts and adipocytes (Fig. 4E,F). However, a fraction of SNAP-23 is relocated at the plasma membrane after differentiation, suggesting the acquisition of a new function for this protein in adipocytes in parallel to the insulin-dependent GLUT4 translocation process.

4. Discussion

The 3T3-L1 preadipocyte cell line has been widely used to study the cellular and molecular mechanism of insulin-dependent translocation of the glucose transporter GLUT4 [4,8]. The ability of 3T3-L1 adipocytes to perform GLUT4 translocation depends, at least in part, on the presence of a set of proteins that mediate the late steps of this process. Accordingly, gaining of insulin-dependent glucose uptake ability would depend not only on the expression of the specific glucose transporter GLUT4 but also on the presence of the proteins involved in the fusion of intracellular vesicles containing GLUT4 with the plasma membrane. This could indicate changes in the protein expression during adipocyte differentiation that can be observed by silver staining of acrylamide gels of differentiated and non-differentiated 3T3-L1 cells (Fig. 1). In this work, we have used Western blot to further identify and analyze the expression of some of the proteins that have previously been involved in GLUT4 translocation [4,8]. This study has mainly been focused on members of the SNARE category of proteins that, together with other proteins [8], are required for membrane fusion processes and contribute to the specificity of the process [6,42], in particular the t-SNAREs (also q-SNARE as described [43]) SNAP-23 and syntaxin 4, and the v-SNAREs (also r-SNARE [43]) VAMP2 and cellubrevin. The expression of some of these proteins, in parallel to GLUT4, is significantly increased during the differentiation process of 3T3-L1 cells, i.e. cellubrevin in agreement with previous reports [9] and syntaxin 4. Morphological data also showed increased amounts of both proteins after differentiation. Moreover, syntaxin 4 was clearly detected after differentiation in the plasma membrane, in addition to an intracellular pool, according to its role as t-SNARE in the GLUT4 translocation process [11,12,44,45]. Syntaxin 4 was only found in an intracellular pool in non-differentiated cells; however, the low amount of this protein in the fibroblastic phenotype can be under the detection level of the antibody.

In the particular case of SNAP-23, our results showed that its levels were maintained during differentiation in agreement with a previous report [34]. According to its proposed role as a t-SNARE, SNAP-23 has mainly been located in the plasma membrane together with syntaxin 4. However, immunofluorescence results showed the distribution of SNAP-23 in an internal membrane compartment in non-differentiated cells, basically the Golgi complex as demonstrated by partial colocalization with Golgi 58K protein [46], and not at the plasma membrane. Only after adipocyte differentiation, SNAP-23 was found at the plasma membrane, where it is assumed to do its function as a t-SNARE protein in GLUT4 translocation together with syntaxin 4.

Some other proteins that changed their relative levels after 3T3-L1 cell differentiation were defined. Some of these proteins could be involved in other membrane traffic events than GLUT4 translocation, i.e. syntaxin 2. In addition to decreased levels of syntaxin 2 after differentiation, a second band with higher electrophoretic mobility was also detected by Western blot (Fig. 2B). This result could indicate the appearance of either a new syntaxin 2 isoform [47], or a new pool of phosphorylated protein [48]. In any case, a new function for syntaxin 2 could be assigned in 3T3-L1 adipocyte cells compared to the non-differentiated fibroblastic form. One possibility would be the regulation of GLUT4 translocation machinery by competing with syntaxin 4 in protein interactions. Syntaxin 2 is, together with syntaxin 4, the only syntaxin isoform that interacts with Munc-18c, a protein directly implicated in insulin-induced GLUT4 translocation through its interaction with syntaxin 4 [12,49,50]. A proteolytic product is unlikely because all samples were run in parallel and no additional band was detected in non-differentiated cells and confluent cells in differentiating medium. Curiously, levels of Munc-18 were decreased during differentiation of 3T3-L1 cells. However, the anti-Munc-18 antibody used in this study can cross-react with several Munc-18 isoforms, and we cannot conclude which Munc-18 isoform levels are affected by the differentiation process in 3T3-L1 cells.

Levels of β_1 Na^+/K^+-ATPase subunit were also decreased after 3T3-L1 differentiation into adipocytes. This result is in agreement with the decreased mRNA levels for either α_1 or β_1 Na/K-ATPase subunits [51] and with the reduced levels of Na^+/K^+-ATPase α_1 subunit [9] previously found during 3T3-L1 cell differentiation into adipocytes.

The levels of α-tubulin were also decreased during 3T3-L1 differentiation in agreement with previous reports on α- and β-tubulin mRNA reduction [52,53] that was implicated in the development of the adipocyte morphology. However, the microtubule cytoskeleton is required for optimal trafficking of glucose transporter contained in the GLUT4 storage vesicle pool [54]. This indicates that the remaining microtubules have a more specific specialized role in the GLUT4 translocation.

In addition to the differentiation process, changes in protein expression in 3T3-L1 cells have been described in other circumstances. Elevated v- and t-SNARE protein levels in 3T3-L1 cells (cellubrevin, VAMP2 and syntaxin 4) are associated with insulin resistance in skeletal muscle and this increase may be reversed by restoration of glycemic control [55]. Moreover, in full-differentiated 3T3-L1 cells, elevated levels of SNARE proteins were observed after a chronic treatment with insulin, while GLUT4 levels were reduced [55]. These results directly suggest that the levels of proteins involved in insulin-dependent GLUT4 translocation can be altered depending on the functional state of the cell.

All together, these results show changes in the expression and cellular distribution of proteins directly involved in insulin-induced GLUT4 translocation during adipose cell differentiation, and support that alterations of this process (i.e. insulin resistance) could be directly related with changes in their expression levels. In addition to changes in the expression of SNARE proteins, other proteins and mechanisms have been directly implicated in the incorporation of GLUT4 into plasma membrane [8]. More work is required to further characterize and correlate the molecular machinery involved in this process.

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