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Role of FAP48 in HIV-Associated Lipodystrophy

Vincenzo Esposito,[1*] Lucrezia Manente,[2] Angela Lucariello,[2] Angelica Perna,[2] Rosaria Viglietti,[1] Miriam Gargiulo,[1] Roberto Parrella,[1] Giovanni Parrella,[1] Alfonso Baldi,[3] Antonio De Luca,[2] and Antonio Chirianni[1]

[1]*Third Division Cotugno Hospital, Naples, Italy*
[2]*Department of Medicine and Public Health, Second University of Naples, Naples, Italy*
[3]*Department of Biochemistry, Section of Pathology, Second University of Naples, Naples, Italy*

ABSTRACT

The highly active antiretroviral therapy (HAART) can cause a metabolic syndrome consisting of lipodystropy/lipoatrophy, dyslipidemia, and type 2 diabetes mellitus with an increased cardiovascular risk. The pathogenetic bases of HAART-associated lipodystrophy are poorly known. A genetic screen was used to evaluate proteins that are modulated in HIV-1-infected patients with or without lipodystrophy syndrome, that are routinely treated with HAART regimens. The most significant modulation was represented by FAP48 expression. Stable over-expression of FAP48 was able to alter, in vitro, adipogenesis, acting both on calcineurin and glucocorticoid pathways. Finally, we demonstrated that FAP48 over-expression was able to influence the capacity of some HIV drugs, Saquinavir and Efavirenz, but not Stavudine, Amprenavir, and Indinavir to inhibit adipocyte formation. In conclusion, this molecule could be a potential target for novel therapeutic approaches to the HAART related lipodystrophy in HIV patients. J. Cell. Biochem. 113: 3446–3454, 2012.

KEY WORDS: HAART; HIV INFECTION; LIPODYSTROPHY; FAP48

The introduction of highly active antiretroviral therapies (HAART) has significantly changed the clinical course of HIV disease, with prolonged survival and better quality of life for HIV infected patients. However, this successful therapeutic advance has been partially marked by the development of serious long-term side effects including metabolic alterations, cardiovascular disease, kidney impairment, bone alterations, and adipose tissue redistribution. This last phenomenon is currently indicated as HIV related lipodystrophy [Barbaro, 2006].

Lipodystrophy syndrome includes three clinical conditions characterized by abnormal body fat distribution: lipoatrophy, lipoaccumulation, and a mixed syndrome. To date, there is no universally accepted definition of lipodystrophy. This explains the difficulty in determining prevalence, etiology, and treatment of fat distribution abnormalities that can occur together with other metabolic complications, causing a metabolic syndrome consisting of lipodystrophy/lipoatrophy, dyslipidemia, and type 2 diabetes mellitus, with an overall increased cardiovascular risk of about 1.4 cardiac events/1,000 years of therapy [Barbaro, 2006; Boyd and Reiss, 2006]. Indeed, there are no current guidelines for the treatment of fat distribution abnormalities that occur in the absence of other metabolic complications [Boyd and Reiss, 2006]. The pathogenetic bases of HAART associated lipodystrophy are, then, still poorly known.

In the last years the attention of the researchers has been targeted to the implication of mitochondrial function and stress metabolic enzymes impairment in the development of lipodystrophy [Boyd and Reiss, 2006]. Preliminary reports showed that HAART regimen can affect the quantity of mitochondrial DNA even in the very early stages of the therapy and that adipocyte differentiation can be consequently altered [Barbaro, 2006; Boyd and Reiss, 2006].

Whole genome expression analysis using cDNA microarrays has recently generated some relevant contributions to the knowledge of the genetic bases of alterations of adipocyte differentiation and lipid metabolism following exposure to antiretroviral therapy. A study by Pacenti et al. [2006] showed that, under standard adipogenic differentiation protocols, treatment with antiretroviral drugs modulated the expression of genes that play a key role in the determination of the adipocyte phenotype and genes involved in lipid metabolism and cell cycle control.

Even if some studies suggested an independent role for HIV in the development of lipodystrophic phenotype, there is a widely accepted

*Correspondence to: Dr. Vincenzo Esposito, MD, PhD, Terza Divisione di Malattie Infettive, A.O. Cotugno, via G. Quagliariello 54-80131 Napoli, Italy. E-mail: esposvin@libero.it
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consensus that the risk to develop fat redistribution in HIV patients has to be mostly related to antiretroviral therapy. More in detail, lipoatrophy that affects the face and extremities has been linked to the exposure to NRTI drugs, while PI exposure has been mainly associated with central fat accumulation. More recently, it has been reported that Efavirenz, an antiretroviral compound belonging to the NNRTI class, is associated with an increased risk of lipoatrophy [Zhang et al., 2008].

Actually, both peripheral fat loss and central fat accumulation often coexist in the same patients, probably because HAART regimens usually include two different classes of antiretroviral drugs (NNRT + PI or +NNRTI). However the impairment of adipocyte differentiation seems to be such a complicated issue that one can not exclude the possibility that derangement of one or more genes induced by the exposure to more than one class of antiretroviral compounds could cause mixed metabolic alterations (dislipidemia and diabetes) as well as mixed lipodystrophic clinical pictures.

In order to investigate new pathways involved in the development of lipodystrophy, our group already reported an array screening performed using two identical filter arrays with cDNA-labeled probes, generated from the adipose tissue of either HIV patients affected or not affected by lipodystrophy [Esposito et al., 2012]. Among the genes selected, we focused our attention on a recently described 48 kDa protein of 417 amino acids named FAP48.

The FAP48 sequence contains a high level of hydrophobic residues, namely leucine, and expression of its gene appears ubiquitous. FAP48 does not have any homologies with already known proteins except with its splice variant FAP68 [Chambraud et al., 1996; Grisendi et al., 2001]. FAP48 interacts with FK506-binding proteins (FKBPs) such as FKBP52 and FKBP12, which belong to the large family of immunophilins. The main cytoplasmic FKBP isoform is FKBP12, which, in a complex with FK506, binds to and inhibits the phosphatase calcineurin (CaN). Subsequently, dephosphorylation and nuclear translocation of nuclear factor of activated T cells (NFAT) can no longer occur. NFAT proteins are transcriptional factors for a variety of genes and play key roles, influencing the development and adaptation of numerous mammalian cell types [Horsley and Pavlath, 2002]. NFAT proteins also appear to regulate adipocyte differentiation in vitro [Ho et al., 1998]. FKBP52 is another member of the FKBP family that was originally found associated with heat shock protein with a molecular mass of 90 kDa (HSP90) in the hetero-oligomeric forms of steroid receptor complexes.

Drawing from this background we decided to investigate the molecular mechanism able to influence adipocyte differentiation by FAP48. In particular we evaluated the effects of the over-expression of FAP48 on the complex FAP48-FKBP.

MATERIALS AND METHODS

POPULATION STUDY

Eight patients routinely followed at the Third Division of Infectious Diseases of the A.O. "Cotugno Hospital" of Naples, Italy, were included in the study. The diagnosis of lipodystrophy was obtained clinically by two independent observers (V.E. and M.G.) according to the antiretroviral therapy-associated Lipodystrophy European Comparative Study ALECS Group classification [Galli et al., 2000] and a 100% inter-observer level of concordance was reached. Following the approval of the ethical committee, all of them signed the informed consent for the treatment of personal clinical and laboratory data and accepted to give a adipose sample for genetic researches. Information regarding any previous diseases including metabolic alterations and familiarity as well as medical history were obtained from the database of the clinical division. Patients with any metabolic disorder or a documented familiarity for metabolic diseases prior to the diagnosis of HIV infection were not enrolled in the study.

NORTHERN BLOT ANALYSIS

Human adipose tissue obtained during plastic surgery from four patients naïve to antiretroviral therapy with HIV diagnosis performed at minimum 12 months and from four patients treated by the HAART association regimen including protease inhibitors (PIs) affected by lipodystrophy, already used for arrays experiments were, then, quantified spectrophotometrically and their integrity was confirmed by fractionation of 1 μg of RNA on 1% agarose gel with ethidium bromide staining. Ten micrograms of RNA from each of the two different pools were subjected to electrophoresis through a 1% denaturing agarose gel containing formaldehyde. RNAs were transferred overnight onto a Hybond-N+ (Amersham, Milan, Italy) nylon membrane with 20× SSC and RNA was UV-crosslinked onto the membrane. The membrane was then hybridized at 68°C using the ULTRAhybTM hybridization buffer (AMBION) with [32P]dCTP random primer labeled cDNA probes (Random Primed DNA Labeling kit; Boeheringer Mannheim) using 1×10^6 cpm/ml. Filters were probed with FAP48 and with β-actin to normalize the signals detected. After overnight hybridization, blots were washed in 2× SSC/0.1% SDS twice at room temperature for 10 min, then in 0.1× SSC/0.1% SDS three times at 68°C for 20 min and exposed to a Kodak X-ray film at −80°C with the aid of an intensifying screen. Two different RNA preparations from each sample were utilized and the different patterns of expression observed were confirmed in duplicate.

CELL CULTURE, PLASMID PREPARATION, AND STABLE DNA TRANSFECTION

3T3-L1 preadipocytes were cultured and differentiated with standard protocol as described previously [Esposito et al., 2009]. Briefly, 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in humidified atmosphere of 5% CO_2 95% air at 37°C to confluence. Two days later, the induction of adipocyte differentiation was initiated by treatment of the cells with the differentiation medium containing 1 μM insulin, 1 μM Dexamethasone, and 0.5 mM isobutylmethylxanthine for 2 days, followed by 2 days of treatment with the medium containing 1 μM insulin alone. Medium was replaced every 2 days for the following 6 days. Stable transfected 3T3-L1 cells were generated with mammalian expression vector for pcDNA3-FAP48 and pcDNA3 empty vector as control using PolyFect Transfection Reagent (QIAGEN) according to the manufacturer's instruction. Stably transfected cells were selected with 800 μg/μl G418. The pcDNA3-FAP48 was generous gift by Dr. Beatrice Chambraud.

RT-PCR (REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION) ANALYSIS

Total RNAs, isolated by High Pure RNA Isolation Kit (Roche Diagnostics, Milan, Italy) from stably transfected 3T3-L1 cells with empty vector and pcDNA3-FAP48, were transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche Diagnostics) at 42°C for 45 min according to the manufacturer's instructions. Two microliters of complementary DNA (cDNA) were amplified in a reaction mixture containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, and 2.5 units of Taq DNA polymerase (Roche Diagnostics) in a final volume of 25 μl. For the co-amplification conditions PCR was carried out in the presence of 0.5 μM sense and antisense FAP48 (FAP48for GCAAAGAACA-GAAGAGTC, FAP48rev CTGGTTCTCTAAATAATGTAT), PPARγ (PPARfor CTATGAAGACATTCCATTCAC, PPARrev GAGGGAG-TTAGAAGGTTC), aP2, (aP2for GTGGCAGGCATGGCC, aP2rev GTCGTTGAAGGCGGCC), and 0.05 μM sense and β-actin primers. The PCR products were analyzed by electrophoresis on 1.8% agarose gel in TBE. Densitometric analysis of ethidium-bromide-stained agarose gel was carried out by NIH image V1.6 software. The ratio between the yield of each amplified product and that of the co-amplified internal control allowed a relative estimate of mRNA levels in the sample analyzed. The internal control was a housekeeping gene whose PCR product was not overlapping with the interested gene.

PROTEIN EXTRACTION AND WESTERN BLOTTING ANALYSIS

3T3-L1 cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin] for 30 min in ice. Total extracts were cleared by centrifugation for 30 min at 4°C at 10,000 rpm and assayed for the protein content by Bradford's method. Fifty micrograms of protein from each cell lysate were separated by a 8% SDS–PAGE and transferred to PVDF membranes and the filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. The membranes were probed with the primary antibodies against: PPARγ, aP2, NFAT, ERK, p-ERK, FKBP52, Hsp90 (Santa Cruz Biotechnology, CA), and Hsp70 antibody (heat shock proteins) (Santa Cruz, CA) was used to estimate equal protein loading (data not shown). Primary antibodies were incubated following the suggestions of the companies. Then the membranes were incubated with 1:5,000 peroxidase-conjugated anti-mouse immunoglobulins for 1 h at 22°C and 1:2,500 peroxidase- conjugated anti-rabbit immunoglobulins for 1 h at 22°C. They were extensively washed and finally analyzed using the ECL system (Amersham).

RED OIL O STAINING

Intracellular lipid accumulation was determined by Red Oil O staining (Sigma) during adipocyte differentiation as described previously [Esposito et al., 2009]. The cells were washed twice with ice-cold phosphate-buffered saline (PBS), fixed with 4% (w/v) paraformaldehyde in PBS for 20 min and stained with 0.5% (w/v) Red Oil O solution in isopropanol for 1 h at room temperature. After staining the cells were washed with PBS to remove excess stain prior to photography.

IMMUNOPRECIPITATION

Stably transfected 3T3-L1 cells with pcDNA3 empty vector and pcDNA3-FAP48 preadipocytes were differentiated with standard protocol only for 2 days [Esposito et al., 2009]. The assay was performed at day 2 because the treatment with dexametasone induces the glucocorticoid receptor (GR) activation. The choice to perform the experiment only 2 days after the induction was dictated because the long treatment with Dexamethasone is able to stimulate the translocation of GR in the nucleus. Proteins from cell lysates (1 mg) were immunoprecipitated as described previously [Cottone et al., 2006]. Briefly, 3T3-L1 cells were trypsinized and centrifuged at 4°C after 2 days at start differentiation, and the pellet was lysed by incubation in lysis buffer for 30 min at 4°C. The lysate was centrifuged and the supernatant recovered. After a preclearing with 40 μl 50% slurry of protein A-sepharose (Amersham) and 2 μg of purified mouse IgG (Pierce), protein extracts (1 mg) were incubated with antibody against FKBP52 in lysis buffer for 2 h at 4°C. One microgram of purified mouse IgG was used as negative control. Twenty microliters of a 50% slurry of protein A-sepharose were added and incubated for 1 h at 4°C. The sepharose resin was collected by brief centrifugation and subjected to three rounds of washing with 1 ml of lysis buffer per wash. Proteins were resolved by 10% SDS–PAGE and transferred to PVDF membranes (Millipore) in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 5% methanol, and 0.05% SDS). The membranes were blocked with 5% milk in TBS-T buffer (2 mM Tris, 13.7 mM NaCl, 0.1% Tween-20, and pH 7.6), and the blots were incubated with antibodies against FKBP52 and Hsp90, and then washed with TBS-T. The membranes were then incubated with anti-mouse Ig coupled with horseradish peroxidase (Amersham) and washed with TBS-T. The presence of secondary antibody bound to the membrane was detected using the ECL system (Amersham).

CHEMICALS AND TREATMENTS

Indinavir was kindly provided by Merck Sharp and Dohme Ltd (Hoddesdon Hertfordshire UK), and Amprenavir was kindly provided by Glaxo Group Ltd. (Greenfor Middlesex, UK). Saquinavir, Efavirenz, and Stavudine were purchased, respectively, from Roche Farma S.A. (Leganes, Spain), and Bristol-Myers Squibb (Meymac France). Saquinavir, Efavirenz, Stavudine, and Amprenavir were dissolved in Me2SO, while Indinavir was dissolved in H_2O. Drug treatment was performed as described previously [Esposito et al., 2009]. Briefly, 3T3-L1 preadipocytes were cultured and differentiated with standard protocol. The induction of adipocyte differentiation was initiated by treatment of the cells with the differentiation medium and with the drug.

RESULTS

FAP48 RNA IS UP-REGULATED IN RNA FROM PATIENTS AFFECTED BY LYPODISTROPHY

Following our previous cDNA-arrays screening, we decided to focus our attention on FAP48 and we confirmed its up-regulation in the RNA pooled from patients affected by lipodystrophy by Northern blot analysis (Fig. 1A).

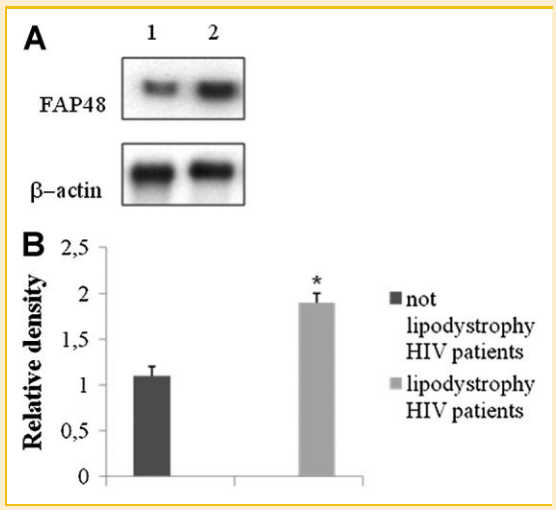

Fig. 1. Fap48 RNA is up-regulated in RNA from patients affected by lypodistrophy Northern blot analysis using FAP48 cDNA probes from pooled RNAs isolated from HIV patients not affected or affected by lipodystrophy (Panel A: lanes 1 and 2, respectively). β-Actin was used as a loading control. Quantitative measurements of the band intensities (Panel B). Data shown are representative of three different experiments (±standard deviation). *Significantly different ($P < 0.05$).

FAP48 OVER-EXPRESSION INFLUENCES THE ADIPOCYTE DIFFERENTIATION

In order to evaluate the role of FAP48 in adipocyte differentiation, we generated a 3T3-L1-FAP48 stable clone. As showed in Figure 2A, the levels of FAP48 mRNA expression were significantly higher in the cells stably transfected with the pcDNA-FAP48 vector respect to the control with the empty vector, as also confirmed by the quantitative measurements of the band intensities (Fig. 2B). Afterwards 3T3-L1 stably transfected cells were cultured and exposed to the treatment with Dexamethasone, Isobutylmethyl-xathine and Insulin for 6 days to obtain a complete differentiation in adipocyte. RNA from 3T3-L1 cells at day 2, 4, and 6 after induction, was extracted and examined by RT-PCR to evaluate the expression of FAP48 (Fig. 2C). The assay demonstrates that FAP48 transcription was present at all points of the time course in 3T3-L1 stably transfected with pcDNA-FAP48 and modulated during adipocyte differentiation.

Instead it waspresent in 3T3-L1 stably transfected with empty vector starting from day 4 and more expressed at 6 day, These data were also confirmed by the quantitative measurements of the band intensities (Fig. 2D).

Next, we compared the expression of PPAR-γ and aP2, two well-known adipogenic markers, by RT-PCR at different times after induction of adipogenesis between the 3T3-L1 cells stably transfected with pcDNA3-FAP48 and the cells stably transfected with empty vector. Figure 3A shows that FAP48 over-expression

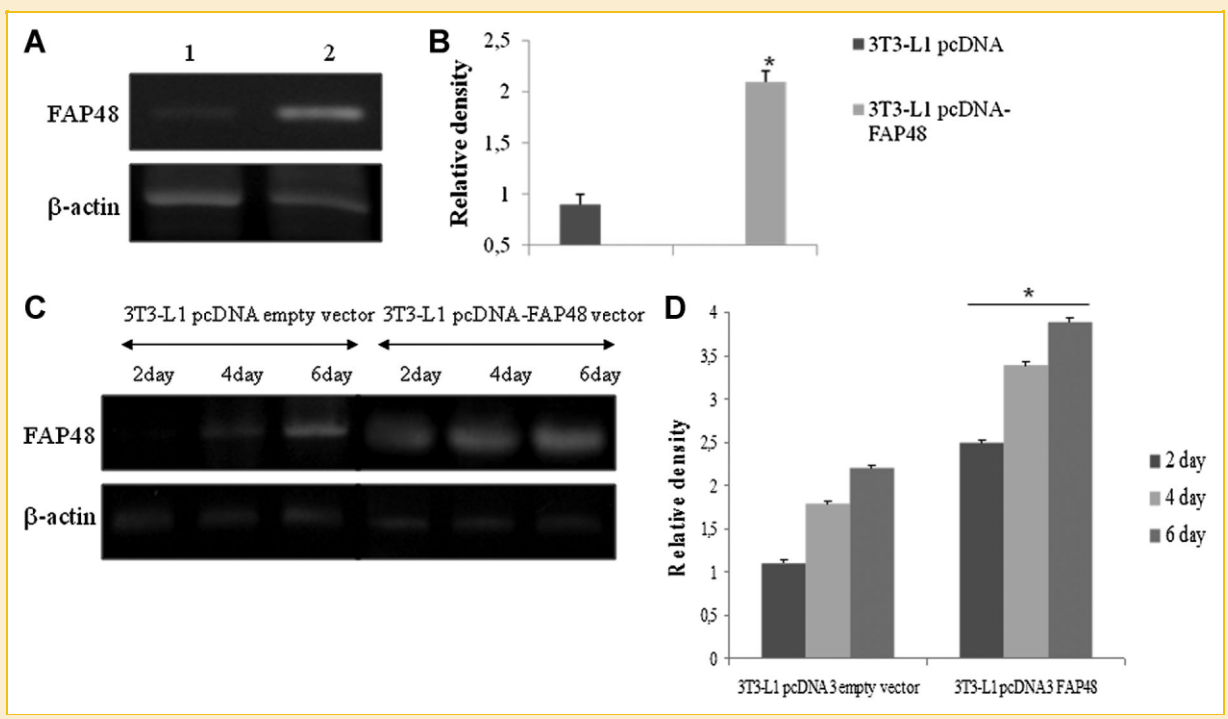

Fig. 2. Panels A and B: (A) Generation of 3T3-L1 cells stably transfected with the pcDNA empty vector (lane 1) and pcDNA-FAP48 vector (lane 2). β-Actin was used as a loading control. (B) Quantitative measurements of the band intensities. Panel C and D: (C) Levels of FAP48 mRNA in 3T3-L1 stably transfected cells with empty vector and pcDNA-FAP48 vector during differentiation. β-Actin was used as a loading control. (D) Quantitative measurements of the band intensities. Data shown are representative of three different experiments (±standard deviation). *Significantly different ($P < 0.05$).

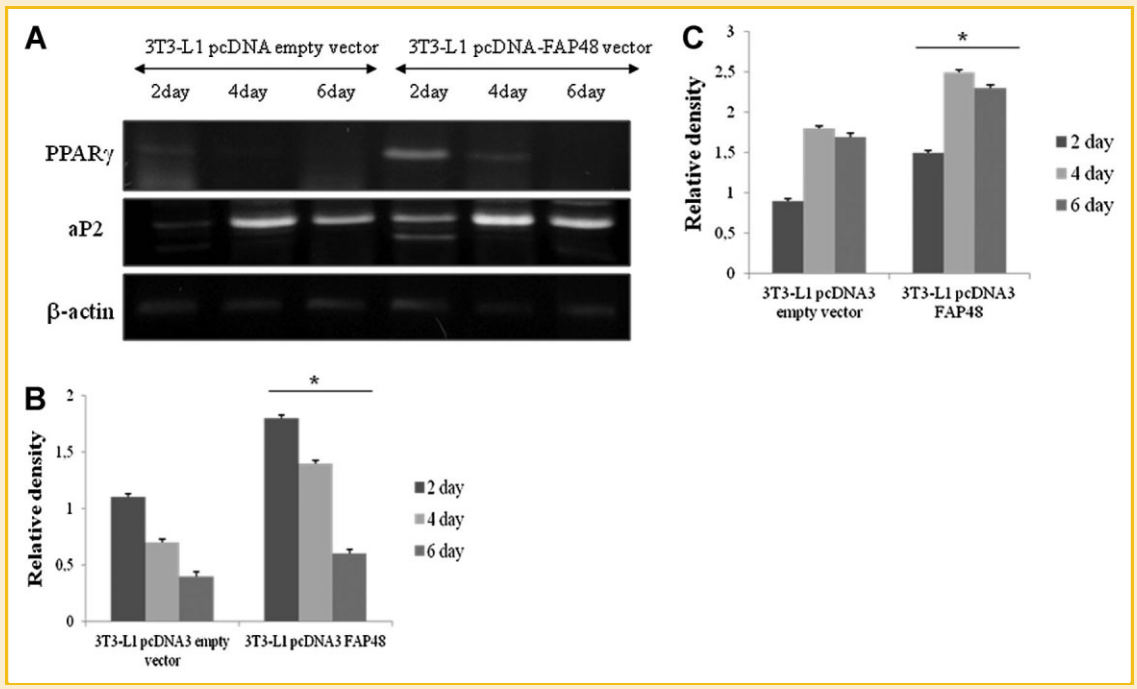

Fig. 3. Fap48 over-expression influences the adipocyte differentiation. Panel A: Expression of mRNA adipocyte markers in stably transfected cells with the pcDNA empty vector and pcDNA-FAP48 vector. β-Actin was used as a RT-PCR loading control. Panel B and C: PPARγ and aP2 quantitative measurements of the band intensities. Data shown are representative of three different experiments (±standard deviation). *Significantly different ($P < 0.05$).

influences the expression of these two proteins during adipocyte differentiation. In detail, expression of PPARγ and aP2 mRNA were up-regulated in the FAP48 transfected cells compared with the control cells. These data were also confirmed by the quantitative measurements of the band intensities (Fig. 3C and D).

The protein expression levels, detected with an immunoblotting, of the adipogenic transcription factors showed that PPARγ and aP2 were up-regulated in the FAP48 transfected cells compared with the control cells (Fig. 4A). These results indicate that FAP48 over-expression in 3T3-L1 preadipocytes positively regulates adipocyte differentiation. Also the C/EBPα expression was analyzed and its trends, was similar to PPARγ (data not shown).

Finally to further confirm the involvement of FAP48 as the adipogenesis regulator, we examined the 3T3-L1 cells stably transfected with pcDNA3-FAP48 and the cells stably transfected with empty vector using Oil Red O staining. As shown in Figure 4B the pre-adipocyte FAP48 stable clone cells showed a increase in oil droplets in contrast to the control cells at the end of the differentiation process.

FAP48 OVER-EXPRESSION RESULTS IN RAPID NFAT DEPHOSPHORYLATION BY ACTIVATING CAN AND PROMOTES ERK1/2 PHOSPHORYLATION

We, next, analyzed the effects of the over-expression of FAP48 on the activity of the complex FAP48-FKBP. In detail we evaluated if over-expression of FAP48 in 3T3-L1 cells was able to modulate the activity of CaN. The best characterized substrate of CaN is a family of transcription factors, the NFAT [Rao et al., 1997]. Therefore, we examined NFAT phosphorylation in 3T3-L1 stably transfected cells with the empty vector and pcDNA-FAP48 vector during adipocyte differentiation. NFAT was dephosphorylated at day 4 in cells transfected with FAP48, in contrast with the control cells where

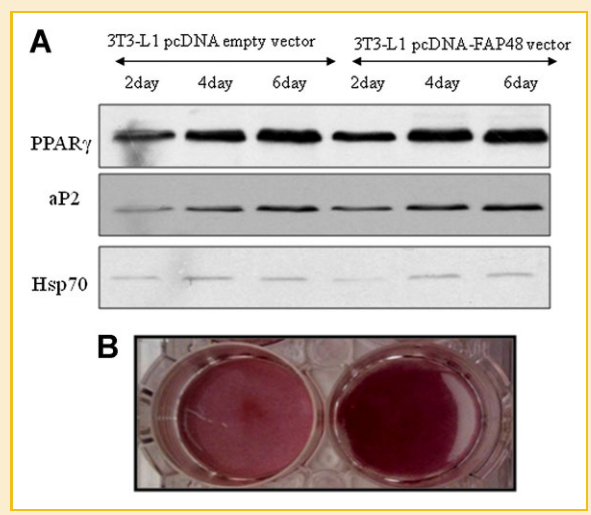

Fig. 4. Fap48 over-expression influences the adipocyte differentiation. Panel A: Expression of proteins adipocyte markers in stably transfected cells with the pcDNA empty vector and pcDNA-FAP48 vector. Hsp70 was used as a western blot loading control. Panel B: Red Oil O staining in stably transfected cells with the pcDNA empty vector (left) and pcDNA-FAP48 vector (right).

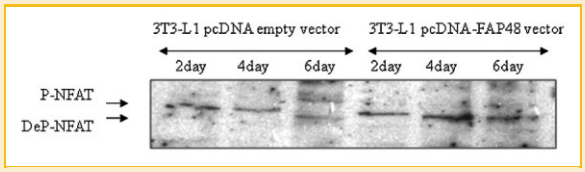

Fig. 5. FAP48 over-expression acts on the NFAT phosphorylation state. Expression of NFAT phosphorylation and dephosphorylation state in stably transfected cells with the pcDNA empty vector and pcDNA-FAP48 vector.

NFAT was dephosphorylated only at the end of differentiation (see Fig. 5) This result suggests that FAP48 over-expression results in rapid NFAT dephosphorylation by activating CaN.

As already showed in Figure 3, aP2 gene transcription, a gene expressed at the last phase of the adipocyte differentiation, was anticipated after over-expression of FAP48. These data support the role of FAP48 in the activation of adipocyte differentiation through a pathway involving NFAT. To investigate whether FAP48 over-expression, through the enhancing of the CaN activity, was able to increase ERK1/2 activation, we performed phospho-ERK1/2 and ERK1/2 immunoblot analysis in the 3T3-L1 preadipocyte stably transfected cells with empty vector and FAP48pcDNA3 during adipocyte differentiation. As shown in Figure 6 phospho-ERK1/2 was detected in the 3T3-L1 preadipocyte stably transfected with FAP48pcDNA3 at day 2 and 4 from the beginning of differentiation compared with the control. Interestingly, the ERK1/2 protein levels did not show particular differences in 3T3-L1 preadipocyte stably transfected cells with empty vector and FAP48-pcDNA during adipocyte differentiation.

FAP48 OVER-EXPRESSION INCREASED THE AMOUNT OF FBP52-HSP90 COMPLEX

To further analyze the effects of the complex FAP48 versus FKBP52 on adipocyte differentiation, we investigated the effects of FAP48 over-expression on FKBP52—GR—HSP90 complex. In detail FKBP52 binds with GR through HSP90 [Ning et al., 2002].

To determine whether FAP48 over-expression was capable to influence this complex, we performed immunoprecipitation with an antibody against FKBP52 followed by immunoblotting with anti-Hsp90 antibody. The experiment, showed in Figure 7 was performed on 3T3-L1 preadipocytes stably transfected with empty vector and

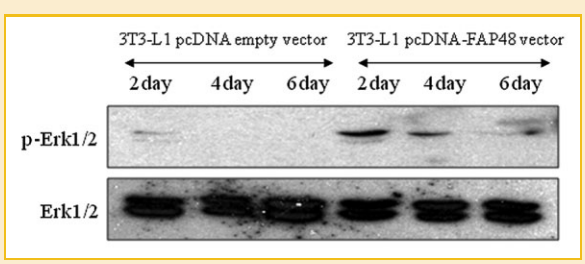

Fig. 6. Expression of ERK signaling in 3T3L1 cells stably transfected with the pcDNA empty vector and pcDNA-FAP48 vector.

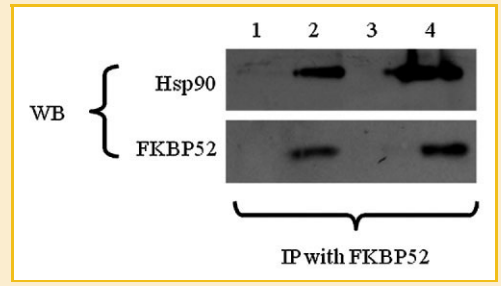

Fig. 7. FAP48 over-expression increased the amount of FBP52-Hsp90 complex. Immunoprecipitation with anti-FKBP52 lane (2 and 4) and NRS (lane 1 and 3) followed by immunoblotting with Hsp90 (upper panel) and FKBP52 (lower panel). Lane 1 and 2 showed 3T3-L1 stably transfected cells with empty vector. Lane 3 and 4 showed 3T3-L1 stably transfected cells with and pcDNA-FAP48 vector.

FAP48pcDNA3, 2 days after the induction of differentiation with Dexamethasone. The choice to perform the experiment only 2 days after the induction was dictated from the observation that long treatment with Dexamethasone is able to stimulate the translocation of GR in the nucleus [Ning et al., 2002].

Immunoprecipitation with anti-FKBP52 followed by immunoblotting with Hsp90 showed that FKBP52 was bound to Hsp90 in both conditions: in 3T3-L1 stably transfected cells with empty vector (lane 2, upper panel) and in the same cells stably transfected with pcDNA3-FAP48 vector (lane 4, upper panel). However, the expression level of Hsp90 that we were able to rescue through the immunoprecipitation followed by immunoblotting was higher in FAP48 stable clone compared to the control cells. We performed, as a control, immunoprecipitation with an antibody against FKBP52 followed by immunoblotting with the same antibody both on 3T3-L1 cells stably transfected with empty vector and pcDNA-FAP48 vector (Fig. 7). This result suggests that FAP48 over-expression increased the amount of FBP52-Hsp90 complex. This complex binds GR and increases the amount of GR that translocates in the nucleus.

FAP48 OVER-EXPRESSION INFLUENCES THE CAPACITY OF THE HIV DRUGS TO INHIBIT ADIPOCYTE FORMATION

We previously characterized, using differentiation markers, the influence of five anti-HIV drugs (Saquinavir, Indinavir, Efavirenz, Stavudine, and Amprenavir) on adipocyte differentiation [Esposito et al., 2009]. Drawing from all the informations published before [Esposito et al., 2009], in the present study, we investigated the effect of the same five anti-HIV drugs, on the 3T3-L1 FAP48 stable clone in order to determine if FAP48 over-expression could influence the capacity of these drugs to inhibit adipocyte formation. We diluted the different anti-HIV drugs into culture medium and cells were induced to differentiate using the standard 6 days protocol. Treatment with Indinavir, Amprenavir and Stavudine did not produce significant differences of expression of the differentiation markers on the 3T3-L1 preadipocytes stably transfected with empty vector and FAP48pcDNA3 (data not shown). Instead, as shown in Figure 8, treatment with saquinavir and Efavirenz promoted and maintained during the 6 day time course an early over-expression of

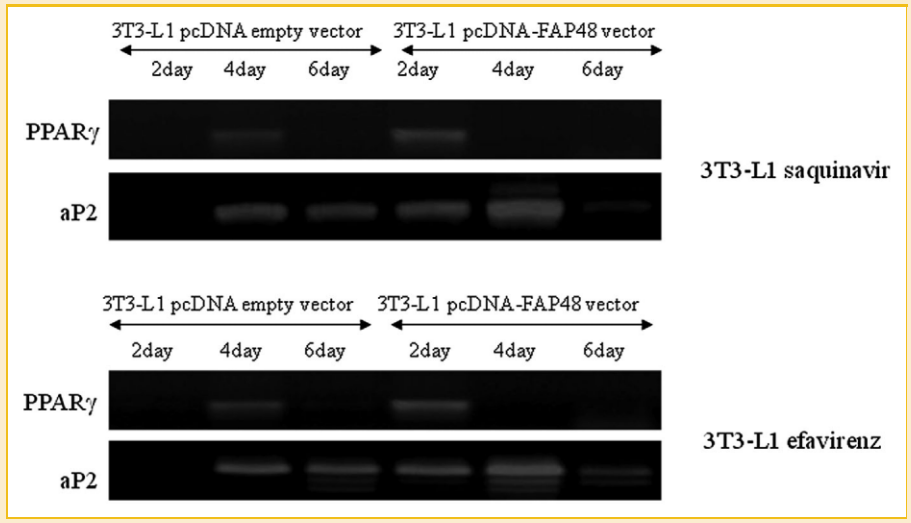

Fig. 8. In FAP48 stable clone the adipocyte markers gene was expressed at 2 day respect to pcDNA empty vector stable clone. Expression of adipocyte markers genes in 3T3-L1 stably transfected cells with the pcDNA empty vector and pcDNA-FAP48 vector after drug treatment.

PPARγ and aP2 in 3T3-L1 FAP48pcDNA3 stably transfected cells, compared with the control cells.

DISCUSSION

In our gene expression assays we identified different expression of genes coding for enzymes and transcription factors involved in lipid metabolism, and regulation of genes coding for immunophilin proteins [Esposito et al., 2012]. These data confirmed previous data from Pacenti et al. [2006] that reported immunophilins family of genes to be regulated by PI and NRTI in differentiating adipocytes. We selected FAP48 for further analysis, among the genes up-regulated in the patients affected by lypodistrophy.

In order to evaluate the involvement of FAP48 in adipocyte differentiation we generated a 3T3-L1-FAP48 stable clone. Experiments on the stable clone showed that FAP48 over-expression in 3T3-L1 preadipocytes positively regulates adipocyte differentiation.

We, next, investigated the molecular mechanism able to influence adipocyte differentiation through FAP48 over-expression. Literature data reported that FAP48 is an endogenous ligand of FKBP and, when complexed to FKBP, could prevent associations between FKBP and other proteins such as CaN [Krummrei et al., 2003]. Indeed, FAP48 interacts with FKBPs such as FKBP52 and FKBP12, which belong to the large family of immunophilins that bind the macrolide immunosuppressant drugs FK506 and rapamycin [Chambraud et al., 1996]. Immunophilins are a family of proteins ubiquitously expressed in two different forms: as free cytoplasmatic species and intracellular receptors acting as coregulatory subunits of molecular complexes including heat shock proteins, GRs and ion channels. Some immunophilins are selectively able to bind to potent immunosuppressants as CsA, FK506, and rapamycin [Galat, 1993]. Immunophilins may be divided into two groups of proteins: the cyclophilin family which selectively binds CsA belongs to the first group, the FKBP family, which binds FK506 or rapamycin with high affinities, belongs to the second group [Galat, 1993]. These two proteins families are enzymes that catalyze cis–trans peptidil-prolyn isomerization and may participate in protein folding in vivo. The drugs binding to these protein families are able to influence their functions [Galat, 1993]. More specifically they are able to inhibit their enzymatic activity [Gething and Sambrook, 1992] and the immunophilin-drug complexes inhibit signal transduction [Cardenas et al., 1994]. The complexes cyclophilin-CsA and FKBP-FK506 inhibit the serine-threonine Ca^{2+}-dependent phosphatase CaN in vitro [Friedman and Weissman, 1991; Liu et al., 1991] and in vivo [Clipsotone and Crabtree, 1992; Foor et al., 1992].

When FKBP interacts with phosphatase CaN interferes with its activity and thereby inhibits NFAT translocation to the nucleus [Emmel et al., 1989] while the dephosphorylate form of NFAT is able to enter the nucleus and activate gene transcription [Flanagan et al., 1991; Northrop et al., 1993].

A role for NFAT in the adipocyte differentiation is also suggested by the fact that NFAT binds to the promoter of aP2 in differentiated 3T3-L1 adipocytes [Ho et al., 1998]. In addition, previous studies demonstrated that NFAT cooperates with C/EBP and regulates PPARγ gene transcription in adipocyte differentiation [Yang et al., 2002; Yang and Chow, 2003]. CaN is a heterodimer that is activated by calmodulin in response to increased intracellular calcium and dephosphorylates the NFAT transcription factor cytoplasmatic subunit, and thereby allows NFAT to enter the nucleus and activate gene transcription [Flanagan et al., 1991; Northrop et al., 1993]. Probably, dephosphorylation induces conformational changes, which then expose nuclear localization sequences and promotes NFAT nuclear accumulation. Inducible and constitutive active protein kinases have been indicated to phosphorylate NFAT, to contrast nuclear accumulation [Beals et al., 1997; Chow et al., 1997].

Therefore, we analyzed the effects of the over-expression of FAP48 on the activity of the complex FAP48-FKBP. Our results

suggest that FAP48 over-expression results in rapid NFAT dephosphorilatyon by activating CaN.

In addition to the calcium mediated NFAT dephosphorylation, a second signaling pathway, the Ras-mediated ERK MAP kinase, is involved in phosphorylation and activation of the NFAT partners. Activation mechanisms include promoting nuclear accumulation, DNA binding, and transcription activation of the NFAT partners. Therefore, in contrast to the negative role of phosphorylation that opposes NFAT nuclear accumulation, phosphorylation promotes NFAT activation by targeting the NFAT partners [Yang et al., 2005]. In addition it has been reported that members of the NFAT transcription factor family are important in aP2 gene regulation [Ho et al., 1998].

Our model showed that (see Fig. 3), aP2 gene transcription, a gene expressed at the last phase of the adipocyte differentiation, was anticipated after over-expression of Fap48. These data support the role of Fap48 in the activation of adipocyte differentiation through a pathway involving NFAT. Moreover the ERK1/2 protein levels did not show particular differences in 3T3-L1 preadipocytes stably transfected with empty vector and FAP48pcDNA3 during differentiation (see Fig. 6).

The cross-talk between ERK1/2 and CaN-NFAT signaling pathways has been suggested by recent reports. For example, mice expressing the activated CaN transgene showed enhanced ERK1/2 activation in the heart [De Windt et al., 2000], while isoproterenol stimulation of cardiac myocytes was shown to activate ERK1/2 signaling through a mechanism involving CaN [Zou et al., 2001]. It has been reported that FAP48 is able to influence the complex between FKBP52–GR–HSP90. In detail FKBP52 binds with GR through HSP90. GR belongs to the superfamily of nuclear hormone receptors. It functions as a ligand dependent transcription factor that regulates different effects of glucocorticoids. In the glucocorticoid action, ligand induces nuclear translocation of GR that regulates gene transcription. In the absence of ligand, GR is located in the cytoplasm as a multiprotein heteroplex that contains heat shock protein Hsp90, Hsp70, and one of the immunophilins, such as the FKBP52 and FKBP51 [Zhang et al., 2008]. Both FKBP51 and FKBP52 are found in mature GR complexes. Hormone binding appears to induce switching of the FKBP52-GR complex, and the GR-Hsp90-FKBP52 heterocomplex shuttles into the nucleus. GR is released into the nucleus, where it regulates the expression of target genes in concert with other transcription factors [Zhang et al., 2008].

In our experimental setting, immunoprecipitation with anti-FKBP52 followed by immunoblotting with Hsp90 showed that FKBP52 was bound to Hsp90 and that FAP48 over-expression increased the amount of FBP52-Hsp90 complex. This complex binds GR and increases the amount of GR that translocates into the nucleus. Taken all together this data confirm the involvement of FAP 48 in the activation of adipocyte differentiation through a pathway involving NFAT.

Our research group evaluated in a previous report [Esposito et al., 2009] five antiretroviral drugs (Indinavir, Amprenavir, Efavirenz, Stavudine, and Saquinavir), belonging to the three main classes of anti-HIV drugs, that were able, in our experimental model, to affect adipocyte differentiation with Efavirenz, Stavudine, and Saquinavir being the most effective drugs in this toxicity. These data were confirmed on this new setting of experiment. However, when we evaluated the expression of PPARγ and aP2 in 3T3-L1 FAP48pcDNA stably transfected cells treated with Saquinavir and Efavirenz an early over-expression of PPARγ and aP2 was promoted and maintained during the 6-day time course, compared with the control cells. This last evidence supports the hypothesis of a protective mechanism, that in 3T3L1 cells could counteract the toxicity of Efavirenz and Saquinavir or could be activated in presence of these drugs.

Drawing from our experimental results it can be then postulated that this mechanism could work trough FAP 48/FBP52/Hsp90 pathway, suggesting this complex as a potential target for novel therapeutic approaches to the HAART related lipodystrophy in patients treated with regimen including Efavirenz and Saquinavir. Further studies are ongoing in our laboratory in order to confirm this suggestive hypothesis.

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