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The growth factor-like adipokine tartrate-resistant acid phosphatase 5a interacts with the rod G3 domain of adipocyte-produced nidogen-2

Christina Patlaka [a], Hong Anh Mai [b], Pernilla Lång [a,*], Göran Andersson [a,*]

[a] Department of Laboratory Medicine, Division of Pathology, Karolinska Institutet, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden
[b] Linköping University, Institute of Technology, Department of Physics, Chemistry and Biology, Linköping University, SE-581 83 Linköping, Sweden

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ABSTRACT

The adipokine tartrate resistant acid phosphatase (TRAP) 5a isoform exerts a growth factor-effect on pre-adipocytes. This study aimed to identify potential TRAP 5a interacting proteins in pre-adipocytes using pull down assays in combination with mass spectrometry. Nidogen-2, a protein shown to be expressed intracellularly and for secretion by pre-adipocytes, was shown to interact, through its globular G3 domain, with TRAP 5a in vitro. In vivo, TRAP 5a interacted with nidogen-2 in cultured 3T3-L1 mouse pre-adipocytes, as well as with transforming growth factor-β (TGF-β) interacting protein (TRIP-1), which is a protein that has previously been suggested to interact with TRAP in bone. In addition, TRAP 5a and nidogen-2 co-localized in adipose tissue cells in situ. These results indicate that TRAP 5a interacts with nidogen-2 and TRIP-1 in pre-adipocytic cells.

1. Introduction

The monomeric 5a isoform of tartrate resistant acid phosphatase (TRAP; EC 3. 1.3.2) or ACP 5 enhances adipocyte proliferation and differentiation in vitro and induces hyperplastic insulin-sensitive obesity when overexpressed in mice [1]. Proteolytic processing of TRAP 5a in an exposed repressive loop domain [2] interacting with the active center results in TRAP 5b. This isoform does not display growth factor activity in MSCs and pre-adipocytes [1], but possesses high phosphatase activity and has been implicated in cell migration [3]. Besides adipocytes, TRAP 5a has been implicated as a growth factor for hematopoietic cells [4] and osteoblasts [5,6]. As a growth factor, TRAP 5a would be expected to interact with extracellular matrix (ECM) proteins, co-receptors and cell membrane receptors. So far only two possible interacting proteins have been identified for TRAP 5a. First, TRAP 5a interacts in vitro with heparan sulfate (HS) and is co-localized with glypican 4 in pre-adipocytes [7] and osteoclast lacuna [8]. Secondly, TRAP has been suggested to interact with transforming growth factor-β (TGF-β) receptor interacting protein (TRIP-1) [5]. In adipose tissue, TRAP 5a is expressed mainly by adipose tissue macrophages (ATMs) [1,9,10] and is secreted into the extracellular matrix (ECM) [9]. Binding of growth factors to ECM is a common feature affecting mainly the availability and cell binding of growth factors [11], e.g. hepatocyte growth factor (HGF) binding to fibronectin and vitronectin [12], both maximizing the cellular response of the ligand-receptor complex.

Since knowledge of TRAP 5a interacting proteins is limited, the aim of this study was to identify potential TRAP 5a interacting proteins in adipose tissue.

2. Materials and methods

2.1. Recombinant proteins

Recombinant human his-TRAP 5a, recombinant human untagged TRAP 5a and recombinant rat TRAP 5a were produced as previously described [7,13]. Recombinant mouse His-tagged nidogen-2 was purchased from R&D systems, Abingdon, UK. Recombinant mouse His-tagged nidogen-2 and fragments G1G2, G2G3 and G3 [14] were a kind gift from Dr. Roswitha Nischt, Department of Dermatology, University Hospital of Cologne, Cologne, Germany.

Abbreviations: ATMs, adipose tissue macrophages; ECM, extracellular matrix; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HS, heparan sulfate; MS, mass spectrometry; PLA, proximity ligation assay; TRAP, tartrate resistant acid phosphatase; TRIP-1, transforming growth factor-β (TGF-β) receptor interacting protein; VEGF, vascular endothelial growth factor.

* Corresponding authors at: F46 Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden. Fax: +46 8 58587730.

E-mail addresses: christina.patlaka@ki.se (C. Patlaka), hong.anh.mai86@hotmail.com (H.A. Mai), pernilla.lang@ki.se (P. Lång), goran.andersson@ki.se (G. Andersson).

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2.2. Cell line, culture and differentiation

3T3-L1 cells (a kind gift from Peter Arner, Karolinska Institutet) were grown and differentiated as previously described [7], with the exception that differentiation was performed for 14 days.

2.3. Pull down assay and mass spectrometry analysis of possible TRAP 5a interacting proteins from pre-adipocytes

Membrane proteins were isolated from 5×10^6 pre-adipocytes (3T3-L1 cells) using Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific, Rockford, IL) according to manufacturer's protocol. For pull down analysis; 1 μg of recombinant human his-TRAP 5a diluted in interaction buffer pH 8.0 (20 mM imidazole (ICN Biomedicals, Irvine, CA), 0.01% Triton X-100 (Sigma–Aldrich), 300 mM NaCl (Merck, Darmstadt, Germany)) was bound to 10 μl NiNTA Magnetic agarose beads (QIAGEN, Silicon Valley, CA) by 1 h incubation in room temperature (RT) under shaking. Purified membrane proteins were then incubated with 1 μg recombinant human his-TRAP 5a bound to NiNTA Magnetic agarose beads in interaction buffer for 1 h. After incubation Ni-beads were placed in a Magna-Sep™ magnetic particle separator (Life technologies) and washed once with interaction buffer pH 8.0 after which human recombinant his-TRAP 5a and TRAP 5a interacting proteins were eluted in elution buffer (250 mM imidazole pH 8.0, 0.01% Triton X-100, 300 mM NaCl). Eluted sample was further analyzed using SDS–PAGE and silver staining as described under Section 2.7. After documentation, selected bands were cut and subjected to in-gel digestion by trypsinization and mass spectrometry on Orbitrap Velos instrument (Fisher Scientific, Gothenburg, Sweden) followed by identity search in Swiss Prot_mouse at the Proteomics Karolinska/Karolinska Institutet.

2.4. Pull down assays

1 μg of human his-TRAP 5a, 1.5 μg His-tagged nidogen-2, 1 μg of domains G1G2, G2G3, G3 or 6X His tag® peptide (Abcam, Cambrige, UK) diluted in interaction buffer pH 8.0 was incubated with 10 μl NiNTA Magnetic agarose beads (QIAGEN)/μg protein for 1 h room temperature (RT) with shaking. 1.7 μg rat TRAP 5a, 250–500 ng of TRAP 5a molecular stoichiometry of 1:1 or 3T3-L1 lysate from 6×10^6 cells, were then added and incubated for 1 h. Ni-bead mixture was placed in Magna-Sep™ magnetic particle separator (Life technologies) and washed once with interaction buffer pH 8.0 after which proteins were eluted in elution buffer. Eluted sample was further analyzed using SDS–PAGE and silver staining as described under Section 2.7.

2.5. Total RNA isolation and cDNA synthesis

Total RNA was isolated from 3T3-L1 cells using RNeasy plus mini kit (QIAGEN) according to manufacturer's protocol followed by quantification (ND-1000 nanodrop spectrophotometer, Thermo Scientific) and quality assessment (Experion system, BIO-RAD, Hercules, CA) with RNA StdSens Analysis Kit (BIO-RAD). Samples with RNA quality indicator (RQI) over 8 were used. cDNA was prepared using Super Script III kit (Invitrogen, Stockholm, Sweden) according to the manufacturer's protocol.

2.6. Real time PCR

1.7 ng cDNA was amplified in doublets (iQ SYBR Green Supermix, CFX96 Real time System, BIORAD) in 10 μl under conditions: 95 °C (3 min), 40 cycles 95 °C (5 s), annealing temp (5 s) and 72 °C (10 s). Melt curves were generated using conditions: 95 °C (10 s), increase of 0.5 °C every 5 s from 65 to 95 °C. Results were analyzed using Bio-Rad CFX manager 2.0 software (BIO-RAD). Primers were as follows: nidogen-2 (forward primer: CGGGT GGCTAACACCGCCAT reverse primer: TGCCGTTGGTGCCCACGTA GA; annealing temperature 60 °C) and endogenous controls 3-phosphoglycerate kinase (pgk-1 forward primer: TAGGGGT TGCATCACTATCATAGG reverse primer: AGTGCTCACATGGCTGA CTTTA; annealing temperature 61 °C) and peptidylprolyl isomerase A (Ppia, forward primer: GGACCAAACACAAACGGTTCC, reverse primer: CACAATGTTCATGCCTTCTTTCAC; annealing temperature 61 °C).

2.7. SDS–PAGE, silver staining and Western blot

Samples were mixed with Laemmli Sample Buffer (BIO-RAD), loaded on Mini-PROTEAN®TGX™ precast gel (BIO-RAD) with running buffer (1 M glycine (Sigma–Aldrich), 17 mM sodium-dodecyl-sulfate (SDS; BIO-RAD) and 124 mM trizma®base (Sigma–Aldrich)) and run according to manufacturer's instructions. Silver staining was performed using PlusOne™ Silver staining kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions.

Samples prepared for interaction of TRAP with TRIP-1, were transferred to PVDF membrane (BIO-RAD) using Mini Trans-Blot® (BIO-RAD) in transfer buffer (25 mM Tris–HCl (Sigma–Aldrich) pH 8.3, 192 mM glycine (Sigma–Aldrich) and 20% methanol (Merck)) according to manufacturer's protocol. Samples from adipocyte cell lysate/media or samples for interaction of TRAP 5a with nidogen-2/nidogen-2 domains, were transferred to PVDF membrane (BIO-RAD) using Turbo Blot system (BIO-RAD) according to manufacturer's instructions.

Membranes were washed 3 * 5 min in TBST (25 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20), blocked in 1% BSA in TBST for 30 min and incubated with rabbit anti-human TRIP-1 (Abcam) diluted 1:500 or rabbit anti-mouse nidogen-2 (Abcam) diluted 1:500 or rabbit anti-rat total TRAP and/or mouse THE™ His tag antibody (GenScript, Piscataway, NJ) diluted 1:2000 and 1:500 respectively in TBST for 1 h at RT or overnight at 4 °C and then washed 2 * 10 min in TBST. TRIP-1 stained membranes incubated with goat anti-rabbit IgG alkaline phosphatase conjugated (Sigma–Aldrich) diluted 1:1000 in TBST for 30 min, washed 2×15 min in TBST and developed using NBT/BCIP (Roche, Basel, Switzerland) diluted in 100 mM Tris–HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$ (Sigma–Aldrich) until color developed and then documented using CanoScan 8800 (CanonTokyo, Japan). Nidogen-2 and TRAP stained membranes were incubated with IRDye 680RD goat anti-rabbit IgG or IRDye 800CW goat anti-mouse IgG (LI-COR Biosciences Ltd., UK) according to manufacturer's instructions and then washed 2 * 10 min in TBST. Visualization/analysis was done using Odyssey CLx Imager (LI-COR Biosciences Ltd.) equipped with Image Studio software (LI-COR Biosciences Ltd.). Colloidal Gold Total Protein Stain (BIORAD) was used for total protein staining according to manufacturer's protocol.

2.8. Staining of nidogen-2 in 3T3-L1 pre-adipocytes

3T3-L1 cells were seeded in 8-well glass chambers (Sigma–Aldrich) fixed in 4% PBS buffered formalin (10 min), permeabilized using 0.1% Triton X-100 (Sigma–Aldrich; 10 min), blocked in 1% BSA in TBST (20 min) and incubated with 10–100 μg/ml rabbit anti-mouse nidogen-2 (Abcam) or 5 μg/ml rabbit IgG control (Santa Cruz Biotechnology Inc., Dallas, TX) diluted at 4 °C overnight. Cells were washed in TBST 2 * 5 min and incubated with goat anti-rabbit Alexa 488 (Invitrogen) diluted 1:100, washed 2 * 5 min in TBST, incubated with Hoescht 33342 (Life Technologies) diluted 1:7500 for 3 min, mounted using Dako's fluorescent mounting media (Dako, Glostrup, Denmark) and analyzed/documented using

Leica DM IRB light microscope (Leica Microsystems, Kista, Sweden) with OpenLab (PerkinElmer, Waltham, MA) or 1AR+ confocal system together with NIS-elements (Nikon, Tokyo, Japan).

2.9. Proximity ligation (PLA) assay

3T3-L1 cells were TRAP 5a treated as described under Section 2.8 with following adjustment: Before fixation cells were treated with 100 nM human untagged TRAP 5a for 1 h at RT and washed (2 * 10 min) with serum free DMEM-F12 media (Life Technologies). After incubation with primary antibodies PLA assay was performed (DuoLink assay kit; Olink, Uppsala, Sweden) according to manufacturer's instructions using PLUS anti-mouse probe and MINUS anti-rabbit probe. Samples were visualized/documented as described in Section 2.8. For quantification, data were analyzed using Volocity demo software (PerkinElmer). For each staining, the area of cell and PLA positive spots were calculated and results were expressed as mean value of spots per megapixel ± standard deviation. Threshold value for intensity was set from untreated cells incubated with primary antibodies for TRAP.

2.10. Immunohistochemistry for nidogen-2 and TRAP in adipose tissues

FVB/N mice (ethical permission Stockholm South Animal Ethical Committee (S5-11) were kept under controlled light/dark conditions with food and water available ad libitum.

Biotinylated rabbit anti rat total TRAP IgG [15] was made using Protein G HP SpinTRAP/Ab spin TRAP (GE Healthcare, Uppsala, Sweden)/HOOK IgG biotinylation kit (G Biosciences, St. Louis, MO). 3.5 μm sections on SuperFrost PLUS (Thermo Scientific) were deparaffinized, re-hydrated, washed in 1 * PBS (Substratavdelningen, Karolinska University Hospital Huddinge, Huddinge, Sweden), treated in 1 mM EDTA (15 min) (Philips Whirlpool M760; Philips, Eindhoven, Netherlands) power 10 and cooled (15 min), washed in TBST 5 min and blocked in 1% BSA (Sigma–Aldrich) (20 min). Sections were incubated with rabbit anti-mouse nidogen-2 (Abcam) diluted 1:100 in 0.1% BSA at 4 °C overnight, washed 3 * 10 min in TBST and incubated with goat anti-rabbit Alexa 568 1:250 (Invitrogen) (30 min). After washing, 4 * 60 min in TBST, section were incubated with biotinylated rabbit anti rat total TRAP 326 μg/ml over night at 4 °C, washed 3 * 10 min in TBST and incubated with Streptavidin complex ALEXA 488 0.02 mg/ml in 0.1% BSA (30 min; Invitrogen) and washed in 3 * 10 min in TBST. Slides were mounted using DuoLink In Situ Mounting Media with DAPI (Olink), visualized/documented using 1AR+ confocal system equipped with NIS-elements (Nikon) software. For co-localization analysis Volocity Demo software (PerkinElmer) was used. Five regions of interest (ROI) were selected and Pearson's global correlation together with overlap coefficients for each channel (M1 and M2) were generated and presented as a mean value ± standard deviation (SD). Threshold was set by sections stained exclusively for TRAP, were no co-localization should occur.

3. Results

3.1. Detection of nidogen-2 as a potential TRAP 5a interacting protein

SDS–PAGE analysis of complexes between TRAP 5a and membrane proteins, using pull-down assays, showed bands at 35, 80, 100 and 150 kDa containing potential TRAP 5a interacting proteins (Fig. 1A). Mass spectrometry (MS) analysis of these bands detected the protein nidogen-2 as a possible TRAP 5a interacting protein. Although keratin, serum albumin and other proteins were also detected, the same nidogen-2 peptide, AITVDPIR located in the rod G3 domain of nidogen-2, was present in all bands analyzed. We therefore, considered nidogen-2 as a potential TRAP 5a interaction protein even though the coverage was low (0.6%) and proceeded with pull-down assays for further interaction studies (below).

3.2. Nidogen-2 is expressed by and secreted from differentiating pre-adipocytes

If nidogen-2–TRAP 5a interaction occurs in adipose tissue then nidogen-2 has to be expressed in adipose tissue. To elucidate if this is the case, expression of nidogen-2 mRNA and protein during adipocyte differentiation were studied. Nidogen-2 mRNA is expressed in pre-adipocytes in detectable levels (Fig. 1B and Supplementary Fig. S1) and increases about five times during adipocyte differentiation (Fig. 1B). Full length nidogen-2 protein was present in both cell lysate and media (Fig. 1C). However, nidogen-2 seems to be proteolytically processed into smaller isoforms (Fig. 1C) as previously observed for nidogens [16]. Intracellularly, nidogen-2 was found in vesicle-like structures and on fiber-like structures in the cytoplasm of pre-adipocytes (arrows Fig. 1D). These data indicate that nidogen-2 is present in adipose tissue and in adipocytes, thus a potential TRAP 5a-nidogen-2 interaction could be physiologically relevant.

3.3. TRAP 5a interacts with the rod G3 domain in nidogen-2 and with TRIP-1 in vitro

To establish if TRAP 5a interacts with Nidogen-2 we used pull-down analysis of TRAP 5a with full-length nidogen-2 or domain specific fragments. TRAP 5a interaction with the suggested TRAP interacting protein TRIP-1 [5] was studied using the same technique. Pull-down analysis with recombinant nidogen-2 and TRAP 5a showed that mouse His-tagged nidogen-2 pulled down rat TRAP 5a (Fig. 2A; lane 2, arrows) and that recombinant His-tagged domains G2-G3 and G3 pulled down non- tagged recombinant human TRAP 5a while no TRAP 5a could be detected when using His-tagged G1-G2 domains as bait (Fig. 2B). Domain G1G2, did not seem to run to the expected size, probably due to the elution procedure, but since the fragment is purified there is a low probability as to not identifying the correct fragment. Also, TRIP-1 was shown to be expressed by pre-adipocytes and his-TRAP 5a was able to pull down TRIP-1 present in a cell lysate from pre-adipocytes (Fig. 2C). Bioinformatics analysis of the protein sequences for nidogen-2 and TRIP-1 revealed that the two proteins do not share any common conserved domains or amino acid sequence similarities (data not shown). These data indicate that TRAP 5a interacts in vitro with the G3 domain of nidogen-2 as well as with TRIP-1 as previously shown [5].

3.4. TRAP 5a interacts with nidogen-2 and TRIP-1 in pre-adipocytes

Having shown that TRAP 5a interacts with nidogen-2 and TRIP-1 in vitro, we investigated if this interaction also occurs in situ in pre-adipocytes treated with TRAP 5a using proximity ligation assay (PLA). In cells treated with TRAP 5a, both TRAP 5a-nidogen-2 and TRAP 5a-TRIP-1 showed significantly increased number of interaction spots compared to untreated cells (Fig. 3A). This showed that both TRAP 5a-nidogen-2 and TRAP 5a–TRIP-1 could interact or at least be localized in close proximity in adipocytes.

3.5. TRAP 5a co-localizes with nidogen-2 in mouse adipose tissue

Lastly, we investigated if TRAP 5a and nidogen-2 was co-distributed in mouse adipose tissue indicating that this interaction could take place also in vivo in adipose tissue. Analysis of mouse adipose tissue showed that nidogen-2 and TRAP were co-localized in what

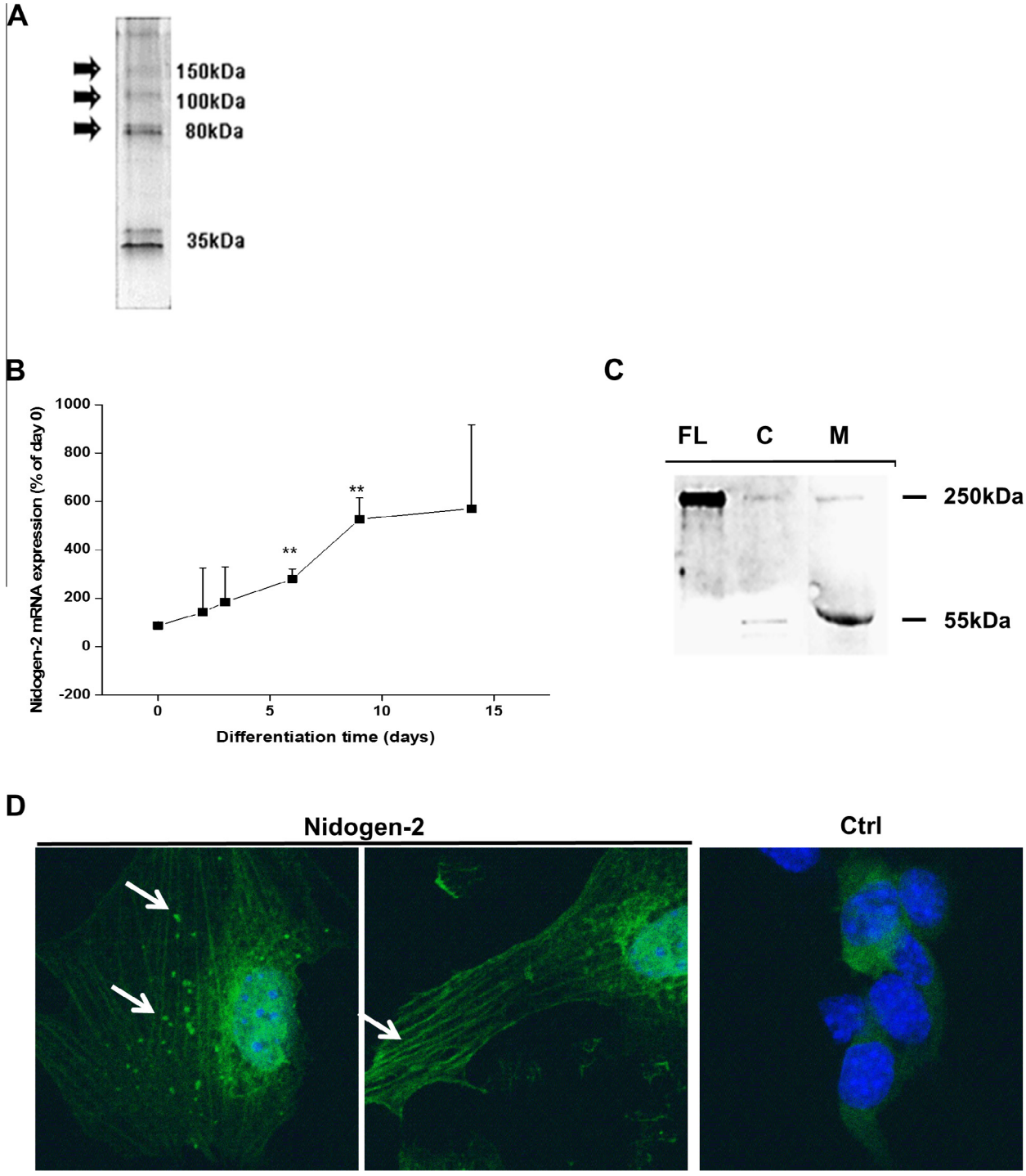

Fig. 1. Screening for TRAP 5a interacting proteins and nidogen-2 expression in adipocytes. (A) Silver stained SDS–PAGE of eluted proteins after pull down assay where Ni-bead bound human his-TRAP 5a was incubated with membrane proteins from pre-adipocytes. Arrows indicate bands analyzed with mass-spectrometry. (B) Nidogen-2 mRNA expression expressed as percentage of mRNA for day 0. Statistical analysis was performed using ANOVA followed by paired *t*-test. **Represent $0.001 \leqslant p$ value $\leqslant 0.01$. (C) Western blot for Nidogen-2 in cell lysate and media of pre-adipocytes on day 0. FL = Full length recombinant mouse nidogen-2 (250 ng) C = Cell lysate of 3T3-L1 pre-adipocytes on day 0, M = Media from pre-adipocytes on day 0. (D) Immunocytochemistry of pre-adipocytes for nidogen-2 (green; left and middle) and control IgG (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appears to be intracellular vesicles (Fig. 3B, arrow), thus indicating that TRAP 5a and nidogen-2 are indeed located partly in the same compartments.

4. Discussion

This study aimed to identify potential TRAP 5a interacting proteins and to establish if TRAP 5a could interact with the suggested TRAP interacting protein TRIP-1 [5] in adipose tissue. Nidogen-2 was detected as a potential interacting protein. The identification of a non-membrane protein among purified membrane proteins could be a consequence of cell-attachment activity of nidogen-2 mediated through its RGD-region [17]. The potential interaction was interesting since TRAP 5a is secreted into adipose tissue ECM by macrophages [9] and ECM proteins are known to affect the distribution, activation and cell binding of growth factors [11].

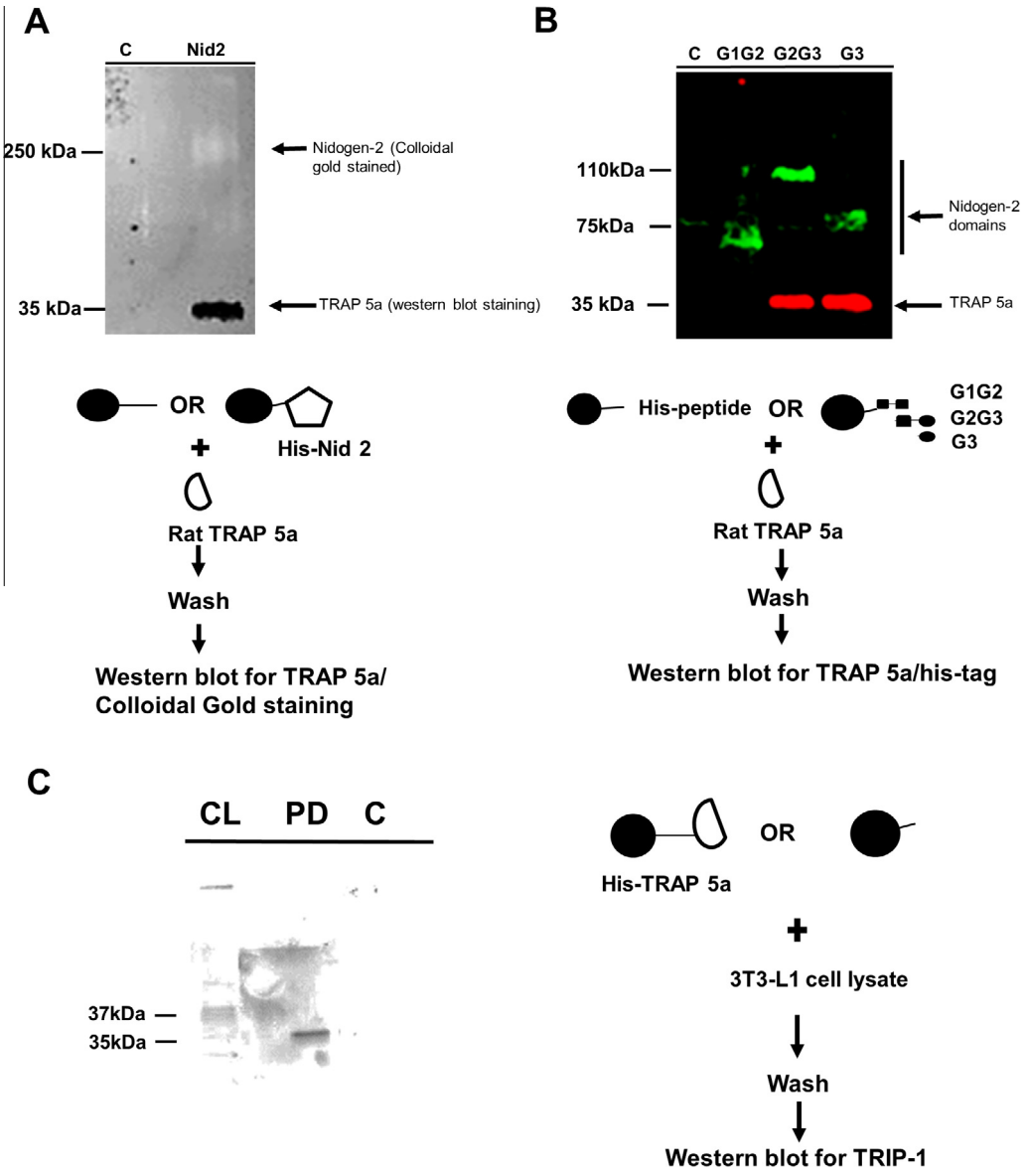

Fig. 2. TRAP 5a interacts with nidogen-2 and TRIP-1 in vitro. (A) Western blot for TRAP and total protein staining (Colloidal Gold; bands demonstrated in white color) after pull down assay using nidogen-2 as bait C = control (Ni-NTA beads without nidogen-2 incubated with TRAP 5a) Nid-2 = Ni-NTA beads with bound His-tagged nidogen-2 incubated with TRAP 5a. (B) Western blot for TRAP 5a (red) and his-tagged domains (green) after pull down assay using His-tagged nidogen-2 domains as bait for rat TRAP 5a. C = Ni-beads with 6X His tag® peptide. (C) Western blot for TRIP-1 on cell lysate of pre-adipocytes and pull down assay CL = TRIP-1 in cell lysate from pre-adipocytes. PD = TRIP-1 after pull down assay where his-TRAP 5a was used as bait. C = Control Ni-NTA beads incubated with cell lysate from pre-adipocytes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TRAP 5a has previously been shown to be synthesized by ATMs [9] and retrieved by pre-adipocytes through endocytosis. In this study nidogen-2 mRNA was shown to be increasingly expressed during adipocyte differentiation in accordance with a previous study [18]. Full length nidogen-2 was present in both cell lysate and media from pre-adipocytes, but the main fraction appeared to be proteolytically processed. Nidogens contain protease cleavage sites [19] possibly explaining these smaller fragments and the presence of the same nidogen-2 peptide in all three bands in the MS analysis. However, the proteolytically processed fragment was not present as a band on the pull down assay of membrane proteins, possibly due to the low abundance of proteins after the pull down assay of membrane proteins. Intracellularly, nidogen-2 appeared in vesicle-like structures in the adipocyte cytoplasm and in fiber-like structures, unlike what has been observed before, where nidogens seemed to be localized pericellularly of adipocytes [18]. Being an ECM protein, previously not reported to exist in the cytoplasm of cells, it was unexpected to find nidogen-2 in the adipocyte cytoplasm, suggesting that it is likely to be present in adipose tissue where also TRAP 5a is present. The coverage of nidogen-2 in this analysis was low and therefore pull down analysis using purified TRAP 5a and nidogen-2 was done to verify the MS data. Full length nidogen-2 as well as a fragment corresponding to the rod G3 domain of nidogen-2 pulled down TRAP 5a in vitro thus verifying TRAP 5a-nidogen-2 interaction through the rod G3 domain of nidogen-2. Suggested TRAP interacting protein TRIP-1 [8] was also expressed in pre-adipocytes and TRAP 5a pulled-down TRIP-1 from adipocyte cell lysate, confirming TRAP 5a-TRIP-1 interaction in vitro. Since nidogen-2 and TRIP-1 interact with TRAP 5a in vitro we studied if this interaction also takes place in situ in TRAP 5a treated adipocytes using PLA assay. In these pre-adipocytes both TRAP 5a - nidogen-2 and TRAP 5a–TRIP-1 interaction

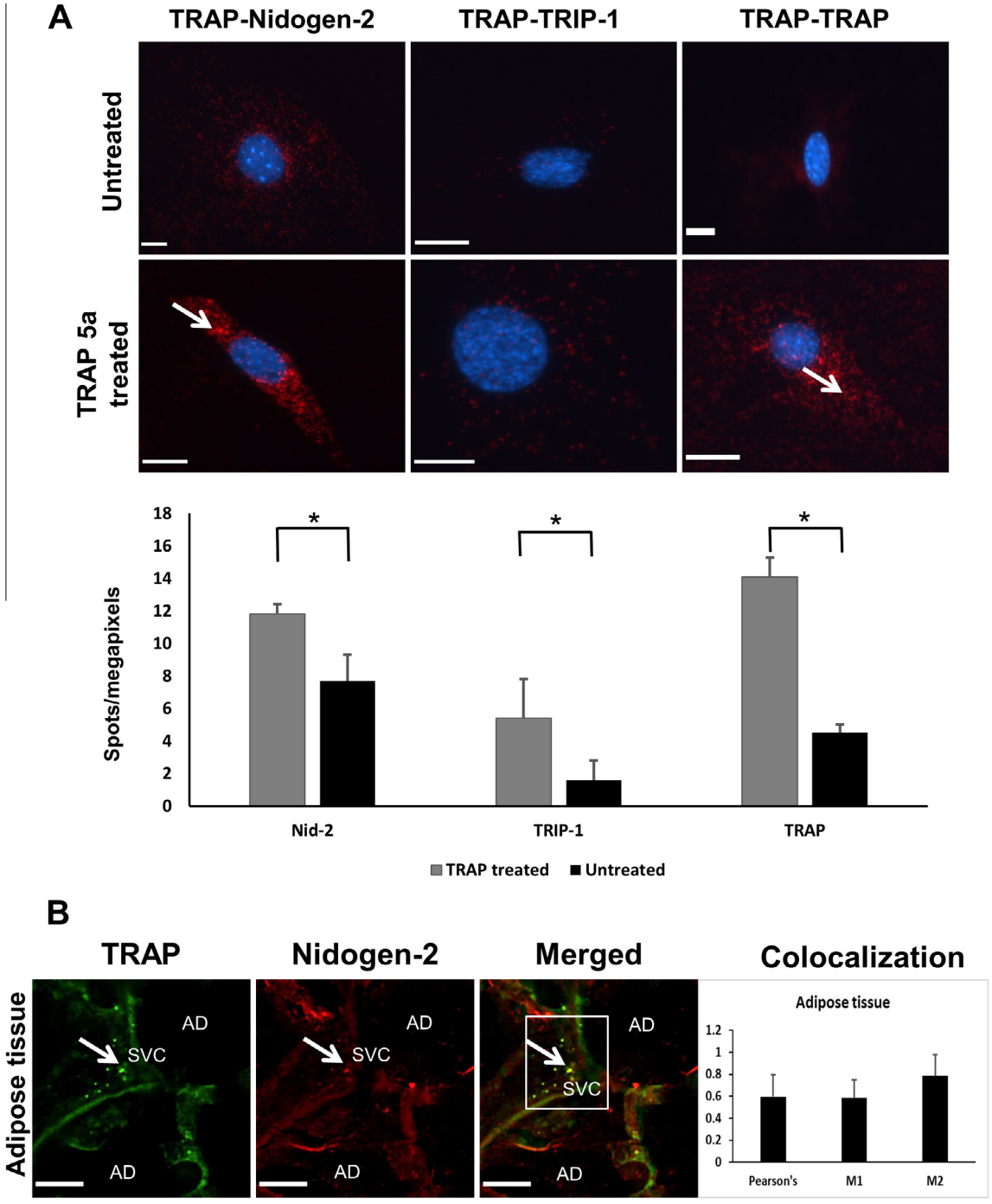

Fig. 3. TRAP 5a interaction with nidogen-2 and TRIP-1 in pre-adipocytes and nidogen-2 and TRAP co-localization in tissue. (A) Proximity ligation assay of untreated (upper panel) and 100 nM TRAP 5a treated cells (lower panel) with rabbit anti-nidogen-2/mouse anti human TRAP 5a (left), mouse anti-human TRAP 5a/rabbit anti-TRIP-1 (middle) and mouse anti human TRAP 5a/rabbit anti rat total TRAP (right). Arrows point at nidogen-2/TRAP 5a interactions spots. Scale bar in all images corresponds to 50 μm. Quantification of PLA assay presented as spots/megapixel. Statistics were performed using Kruskal–Wallis analysis followed by Mann–Whitney test. *Represents $0.01 \leqslant p$ value $\leqslant 0.05$ in 4 cells ($n = 1$). (B) Immunohistochemistry for TRAP (green) and nidogen-2 (red) in adipose tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

occurred. Adipocytes do not synthesize high amounts of TRAP 5a [1,9] but vividly endocytose TRAP 5a which then ends up in the endocytic route in the adipocyte [7]. We hypothesize that the TRAP 5a-nidogen-2 interaction observed in adipocytes takes place in vesicles present along the endocytic route. Nidogen-2 could end up in these vesicles either by endocytosis of TRAP 5a and TRAP 5a bound and/or cell-bound nidogen-2 or it could be redirected to these vesicles instead of secreted. One hypothesis is that nidogen-2 through cell-attachment as well as interaction with TRAP 5a acts to regulate the availability for cell membrane TRAP binding. The observation that TRAP 5a and TRIP-1 interact in adipocytes is surprising since TRIP-1 is located in the cytoplasm/nucleus [20,21] while TRAP 5a carries an ER signal peptide [22] and is also not expressed in adipocytes [1,9] but endocytosed [7]. However, although TRIP-1 lacks a ER signal peptide, osteoblasts have been shown to secrete TRIP-1, and TRIP-1 is present in bone ECM where it has been suggested to be involved in osteoblast differentiation [5,23]. If this would occur also in adipocytes it could explain the

TRAP 5a–TRIP-1 interaction seen in adipocytes as being endocytosed TRAP 5a and TRIP-1 interacting not in the cytoplasm but in ECM, cell-membrane or the endocytosis route.

In summary, apart from the already known binding partner TRIP-1, this study has identified nidogen-2 as a novel binding partner for TRAP 5a in pre-adipocytes. Further, TRAP 5a and nidogen-2 have been shown to be co-localized in adipose tissue cells. These data demonstrate that nidogen-2 and TRIP-1 bind TRAP 5a.

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Disclosure statement

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.112.

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