Cell. Mol. Life Sci. 63 (2006) 939–948
1420-682X/06/080939-10
DOI 10.1007/s00018-005-5551-z


Cellular and Molecular Life Sciences

# Research Article

# Caveolin-1 interacts with the chaperone complex TCP-1 and modulates its protein folding activity

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Received 17 November 2005; received after revision 1 December 2005; accepted 17 February 2006
Online First 29 March 2006

**Abstract.** We report that caveolin-1, one of the major structural protein of caveolae, interacts with TCP-1, a hetero-oligomeric chaperone complex present in all eukaryotic cells that contributes mainly to the folding of actin and tubulin. The caveolin-TCP-1 interaction entails the first 32 amino acids of the N-terminal segment of caveolin. Our data show that caveolin-1 expression is needed for the induction of TCP-1 actin folding function in response to insulin stimulation. Caveolin-1 phosphorylation at tyrosine residue 14 induces the dissociation of caveolin-1 from TCP-1 and activates actin folding. We show that the mechanism by which caveolin-1 modulates TCP-1 activity is indirect and involves the cytoskeleton linker filamin. Filamin is known to bind caveolin-1 and to function as a negative regulator of insulin-mediated signaling. Our data support the notion that the caveolin-filamin interaction contributes to restore insulin-mediated phosphorylation of caveolin, thus allowing the release of active TCP-1.

**Keywords.** Caveolin, TCP-1, folding, insulin, filamin, chaperone

## Introduction

Caveolae are 50–100-nm invaginated plasma membrane domains present in non-lymphoid and neuronal cells. They participate in four main processes, namely, internalization of pathogens and endocytosis of nutriments [1–4], cholesterol transport, signal transduction and cell transformation [1]. Numerous signaling molecules [src kinases, growth factors, G proteins, phospholipase C-$\gamma$, protein kinase-C (PKC) and Ras] have been reported to interact with caveolin-1, a major protein component of caveolae and membrane lipid rafts [5], thus suggesting that these membrane domains play a crucial role in assembling signaling complexes. Caveolin-1 has been shown to function as a tumor suppressor, and phosphorylation of caveolin-1 or reduction of its expression level have been correlated with cell transformation [1, 6]. Caveolin-1 is an integral membrane protein that forms homo-oligomers and shows an unusual hairpin-like structure, exposing both the N and C termini to the cytosol [7]. Functionally, the N-terminal segment of caveolin-1 is of great importance and contains an oligomerization site (residues 61–101) overlapping with a scaffolding domain (residues 82–101), which binds to diverse signaling molecules [5, 8, 9]. Moreover, caveolin-1 residue Y14 is phosphorylated by the insulin receptor (IR) in response to insulin stimulation [10, 11], or by c-Src or c-Abl in response to cell transformation and during oxidative or hyperosmotic stresses [11, 12]. Finally, caveolin-1 is expressed as two isoforms $\alpha$ and $\beta$, the later lacking the first 32 N-terminal residues

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due to alternate initiation of the translation of a single mRNA transcript [13]. The functional difference between the two isoforms remains, however, unclear [7, 13, 14].

TCP-1 is a hetero-oligomeric, double-torus-shaped molecular complex composed of eight different highly conserved polypeptides. It appears as 12–16-nm particles [15, 16], and is abundant in the eukaryotic cytosol. TCP-1 is ubiquitously expressed in all eukaryotic organisms and all mammalian cell types thus far investigated, and is essential for cell survival [17]. TCP-1 assists in protein folding in the cytosol in a process that is Mg dependent and requires ATP hydrolysis [18–20]. In contrast to GroEL, which assists in the folding of a large variety of proteins, TCP-1 folds mainly actin and tubulin and only a limited number of other cytosolic proteins [16, 19, 20]. Although GroEL also binds actin and tubulin, these two proteins undergo their final stage of folding only when associated to TCP-1. TCP-1 identifies actin and tubulin *via* specific subunits of its apical region around the central channel domain and assists actin in reaching its mature conformation by folding its nucleotide binding site [21–23].

In this report, we provide evidence for a specific interaction of TCP-1 and the N-terminal region of caveolin-1 $\alpha$, which hampers the actin folding function of the sequestered chaperone molecules. The phosphorylation of caveolin residue Y14, which has been reported to be involved in several caveolin-mediated signaling pathways including insulin cell activation [10–12], blocks that interaction and restores the function of TCP-1. The fact that the folding activity of TCP-1 is also impaired in cells expressing caveolin Y14F mutant is suggestive for an indirect effect of caveolin on the activity of TCP-1. Using insulin as a cell activation effector, we show that the specific interaction of TCP-1 with caveolin-1 prevents the caveolin-1 from binding and inactivating the cytoskeleton linker filamin known to inhibit insulin-mediated signals transduced by the IR [26, 27].

## Experimental procedures

### Materials

Chemicals were purchased from Sigma (Buchs, Switzerland), except for protein A-Sepharose beads, CNBr-activated Sepharose 4B, and dry polyacrylamide gel strips (pH 3–10; Immobiline™ DryStrip) from Amersham Biosciences (Uppsala, Sweden), fugene 6 from Roche Applied Science (Rotenkreuz, Switzerland), lipofectAMINE2000 from Invitrogen (Frederick, MA), and porcine sequencing grade trypsin from Promega (Madison, WI).

Polyclonal rabbit anti-caveolin antibody (Transduction Laboratories, Lexington, KY), polyclonal rabbit anti-TCP1$\beta$ and monoclonal rat anti-TCP1$\alpha$ (CTA-191) antibodies (StressGen Biotechnologies Corp., Victoria, BC, Canada) were used for immunodetection and immunoprecipitation, respectively. Monoclonal mouse anti-phosphotyrosine (4G10) and polyclonal anti-insulin receptor $\beta$ subunit antibodies were from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody (clone 83–14) to the anti-IR (Biosource International, Nivelles, Belgium) was used for immunoprecipitation. Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human filamin antibody (MAB 1680) was from Chemicon International (Temecula, CA).

### Methods

**Plasmid, cell culture, cell treatment and transient transfections.** HT29 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 µg/ml penicillin and 5 µg/ml streptomycin (GIBCO-BRL Life Technologies Inc., Paisley, UK). HuH7, M2 and A7 cell lines were grown as described previously [24, 25].

The plasmids placIOP-cav-1 and placIOP-mock that allow, respectively, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG)-inducible expression of caveolin-1 or the corresponding mock construct were used for cell transfection as previously described [6]. The Y14 to F mutation in placIOP-cav-1 was introduced by PCR using the QuikChange site directed mutagenesis kit from Stratagene (Gebouw, CA). Dog caveolin $\alpha$ and $\beta$ were inserted after PCR and all inserts checked by sequencing.

HuH7 cells were transiently transfected with caveolin $\alpha$- or $\beta$-encoding plasmids using fugene 6. M2 and A7 cells were transfected by calcium phosphate coprecipitation as described previously [26]. Caveolin expression levels in transfected HuH, M2 and A7 cells were comparable to the physiological expression of caveolin in Swiss mouse 3T3 fibroblasts. However, overexpression of caveolin was obtained by IPTG induction at high concentration. ATP/$MgCl_2$ were both added to the cells at 4 mM for 15 min at 37 °C. M2 and A7 cells were stimulated with insulin as previously described [25], and HuH7 cells were treated with deoxyglucose or insulin at 1 U/ml 2 days after transfection.

**Synthetic peptides and affinity chromatography.** Caveolin-derived peptides (Fig. 1a) were synthesized with an automated solid-phase synthesizer with Fmoc chemistry, purified by reverse-phase HPLC and characterized by mass spectrometry (MS) and amino acid analysis. Peptides were coupled to CNBr-activated Sepharose 4B following the instructions of the supplier.

Fifteen dishes (15 cm in diameter) of HT29 cells were lysed for 5 h on ice in 30 ml 20 mM Tris pH 8.0 containing 1% Brij 78, and the lysate was clarified by centrifugation at 10 000 g for 10 min. The supernatant was ap-

plied overnight at 4 °C to columns containing peptide-derivatized Sepharose. Following extensive washing with lysis buffer supplemented with 0.4 M NaCl, the columns were eluted with 0.1 M glycine, pH 2.4. and the proteins precipitated with chloroform/methanol.

**2D gel electrophoresis and MS identification of proteins.** 2D gel electrophoresis was performed as described [27], with the following modification in the first dimension of the gel: the precipitated proteins were solubilized in 40 mM Tris base containing 7 M urea and 2 M thiourea, 4% CHAPS, 1% Brij 78, 0.001% bromophenol blue and 0.8% resolyte 4–8, 18 mM DTT and 2 mM tributylphosphine. The 2D gels were stained with colloidal silver [28]. The protein spots were excised from the gel, cleaved with trypsin [29] and the generated peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Magic C18 100 µm ×10 cm HPLC column (Spectronex, Switzerland) connected on line to an ion trap Finnigan DecaXP (ThermoFinnigan, CA). The eluting peptides were ionized by electrospray ionization, and the peptide ions detected were automatically selected and fragmented in the ion trap. Individual MS/MS spectra, containing sequence information for a single peptide, were compared with the program TurboSequest [30] against the human subset of the protein sequence database Swiss-Prot 40.36. This resulted in the identification of the peptide and, by association, the protein in the spot.

**Immunoprecipitation, immunoblotting and sucrose density gradient.** Cells were lysed for 4 h on ice in 20 mM Tris pH 8.0 containing 1% Brij 78 and protease inhibitors (9.6 µg/ml benzamidine hydrochloride, 0.9 µg/ml leupeptin and 1.9 µg/ml antipain). An equal amount of protein lysate was immunoprecipitated and analyzed by immunoblotting as described previously [31]. Quantitation of the chemiluminescent signals were performed with ImageJ 1.34s quantification software (NIH, USA). Caveolin-1-enriched fractions were obtained by isolation on sucrose density gradient following solubilization in Triton X-100 as described [7].

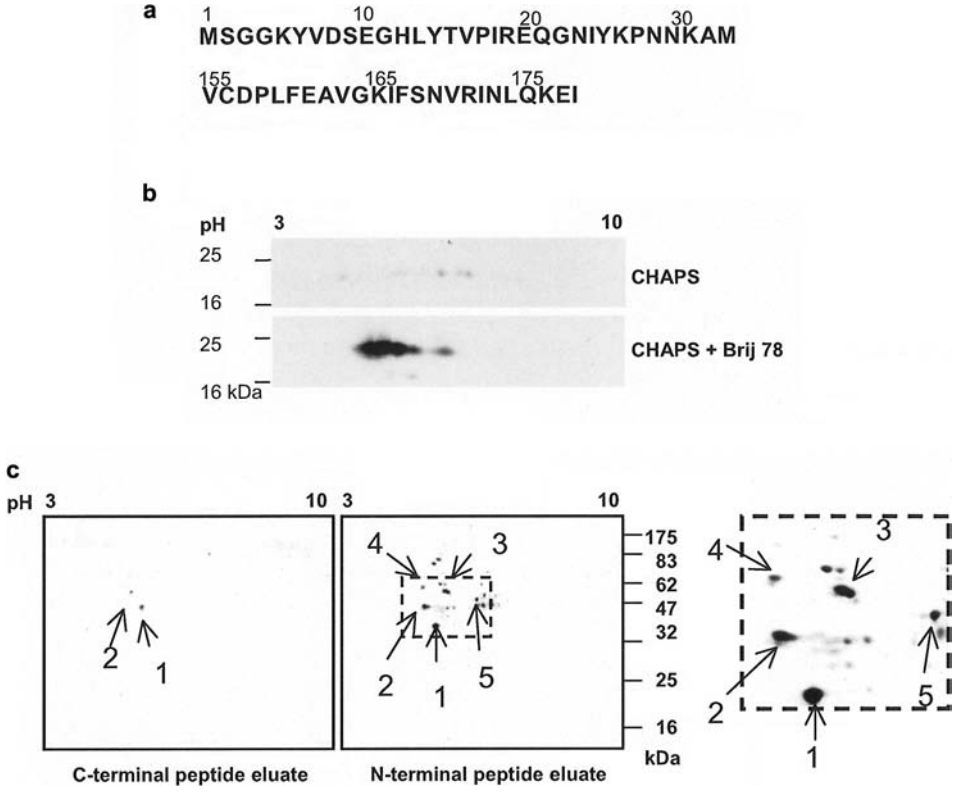


**Figure 1.** Proteins interacting with the N- and C-terminal peptides of caveolin-1. (*a*) N- and C-terminal peptides derived from caveolin-1. (*b*) Low-density detergent insoluble microdomains from caveolin positive Swiss mouse NIH 3T3 cells were isolated by sucrose density gradient. Proteins were resolved by 2D SDS-PAGE using a pH gradient from 3 to 10 in denaturing buffer supplemented either CHAPS or CHAPS/Brij 78 mixed micelles for protein solubilization in the first dimension. Recovery and focalization of caveolin were assessed by Western blotting using caveolin-specific antibodies. (*c*) HT29 cell lysates were passed through N- and C-terminal peptide affinity columns and the eluates were subjected to 2D SDS-PAGE using a pH gradient from 3 to 10 in the first dimension and 12% polyacrylamide gel in the second dimension. The proteins were revealed by silver staining and the right panel shows a blow-up of the N-terminal peptide eluates. Comparable results were obtained in two independent experiments.

**DNase I-inhibition assay**. Following two washes with PBS, the cells were lysed for 4 h on ice in 20 mM Tris pH 8.0 containing 0.3% Triton X-100 and protease inhibitors (45 µg/ml benzamidine hydrochloride, 5 µg/ml leupeptin and 10 µg/ml antipain). The lysate was clarified by centrifugation for 5 min at 10 000 g. The DNase I-inhibition assay was performed as described previously [32, 33] using 2 ml substrate solution containing 0.04 mg/ml DNA and 10 U DNase I. The inhibition was measured by adding 25 µg protein of cell lysate to the substrate solution for 2 min at 26 °C and changes in OD at 260 nm were measured. Dissociation of pre-existing F actin to G actin *in vitro* requires thermal or chemical unfolding of actin [32, 33]. Therefore, in a mixture of filamentous and monomeric actin present in a cell extract, only the monomeric form will inhibit DNase I [32, 33].

## Results

### The N-terminal segment of caveolin-1 $\alpha$ interacts with TCP-1

**Peptide derived from the N terminus of caveolin-1 interacts with TCP-1.** To examine the structural and functional binding properties of caveolin-1 $\alpha$, we used a peptide corresponding to the first 32 N-terminal residues of caveolin-1 $\alpha$ and, as a control, a peptide comprising the last 23 C-terminal residues of caveolin-1 (Fig. 1a). N- and C-terminal synthetic peptides were covalently attached to Sepharose beads and used for affinity chromatography with HT29 cell extracts. Eluted proteins were separated by 2D SDS-PAGE. Due to their resistance to non-ionic detergents, solubilization and recovery of membrane proteins from lipid rafts or caveolae after isoelectric focusing is often of very low efficiency. Good recovery and solubilization of caveolin (Fig. 1b) and of other raft resident proteins such as Lck, Fyn and Thy-1 (data not shown) were only obtained when CHAPS was combined with 1% Brij 78 (see Methods section).

The HT29 cell line was chosen because, as most human colon carcinoma cell lines, it expresses caveolin-1 at very low levels, thus avoiding competition with the immobilized caveolin peptide. A limited number of proteins were specifically eluted from both N- and C-terminal peptide affinity chromatography columns (Fig. 1c). Following trypsin digestion, the resulting peptide fragments were separated and analyzed by LC-MS/MS, and proteins from spots 1, 2, 3 and 5 were identified using the sequence information of the peptide fragments obtained by MS/MS (Fig. 2). Spots 1 and 2 were found in both preparations, although higher amounts were eluted from the N-terminal peptide column. They were shown to contain actin and the mitochondrial ATP synthase beta chain, respectively (Figs 1c and 2). By contrast, spots 3, 4, and 5 were exclusively present in the eluate of the N-terminal peptide affinity column. No protein could be identified in spot 4 and the other detectable spots due to an insufficient amount of material. Unexpected was the identification of spots 3 and 5 as TCP-1 subunits $\theta$ and $\beta$, respectively (Figs 1c and 2). Indeed caveolin has not been reported as being a substrate of the chaperone complex TCP-1. Moreover, using a protein blast search, we did not find any sequence homology between the N-terminal segment of caveolin-1 $\alpha$, or truncated portions of this segment, and any other protein of the uniprot database, suggesting that this interaction might be unique. Finally, among the diversity of proteins interacting with caveolin [5, 9], a comparable association with a molecular chaperone complex has been previously demonstrated with HSP56, cyclophilin 40 and cyclophilin A. Although the complex also involves annexin II, the precise site of interaction remains unknown. The function of this caveolin-chaperone complex is the transport of cholesteryl ester from caveolae through the cytosol to an internal membrane [34, 35].

**Caveolin-1 interacts with TCP-1 in cells.** To determine whether caveolin interacts with TCP-1 in cells, we transfected caveolin-1 $\alpha$ or $\beta$ in human hepatoma HuH or human melanocyte A7 cells that express very low levels of caveolin-1 and 2 [24]. The amount of caveolin expressed in transfected cells was physiological and comparable to endogenous expression of caveolin in Swiss mouse 3T3 cells (data not shown). The level of association of caveolin-1 $\alpha$ to TCP-1 was determined as the ratio between

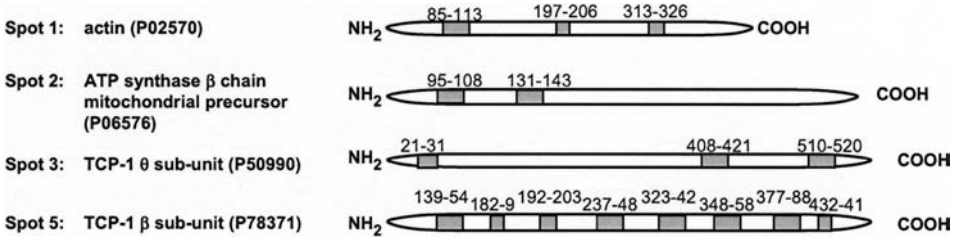


**Figure 2**. The N-terminal segment of caveolin-1 interacts specifically with TCP-1. The protein spots were excised from the 2D gels, digested with trypsin and extracted peptides were separated by capillary LC and sequenced by MS/MS, gray boxes show the peptides for which the sequences were obtained by MS/MS. The proteins were identified with the program TurboSquest searching the Swiss-Prot 40.36 database; their primary accession number is indicated in parentheses.

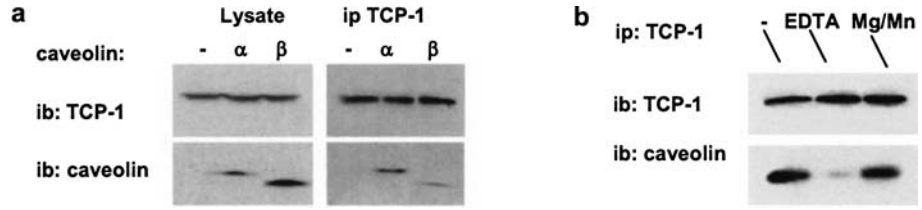


**Figure 3**. TCP-1 associates with caveolin-1 in cells. (*a*) HuH cells untransfected (-) or transfected with caveolin-1 $\alpha$ or $\beta$ were lysed, and the total lysates or immunoprecipitates with anti-TCP-1$\alpha$ antibodies were resolved on SDS-PAGE (12% reducing) and analyzed by Western immunoblotting (ib) with antibodies specific for TCP-1 and caveolin. Caveolin $\alpha$ or $\beta$ lysates and immunoprecipitates shown correspond to the same amount of protein. (*b*) TCP-1 was immunoprecipitated from HuH cells transfected with caveolin-1 $\alpha$ in the presence of EDTA or divalent cations and the immunoprecipitates analyzed as described in (*a*). Shown is one out of three independent experiments.

the amount of caveolin-1 α co-precipitated with anti-TCP-1 antibodies and the amount of caveolin-1 α present in the lysate. Comparison of such quantitations obtained for caveolin-1 α and β indicated that the latter isoform is only marginally associated with TCP-1 since its co-precipitation was always 10 fold lower than the one observed with the α isoform (Fig. 3a). These data confirm the predominant role of the 32 N-terminal residues of caveolin-1 in the interaction with TCP-1, and yet suggests an additional minor site of interaction in caveolin-1 $\beta$. In addition, the interaction was stabilized by Mg and Mn cations and disrupted by chelation of divalent cations with EDTA (Fig. 3b). Finally, TCP-1 immunoprecipitates did not contain caspase 3 nor transferrin receptor, although these proteins were both present in large amount in the cell lysates (data not shown).

To confirm that caveolin interacts with TCP-1 in intact cells, we examined the effect of this interaction on TCP-1 actin folding function. Since caveolin-1 is enriched in caveolae which contain tyrosine kinases and various growth factor receptors (including IR), we stimulated HUH cells with serum, insulin or epidermal growth factor (EGF) that induce caveolin phosphorylation to a variable extent [10, 11]. We measured the impact of these effectors on TCP-1 actin folding activity in the presence or absence of caveolin expression. TCP-1 assists actin in reaching its mature conformation by folding the nucleotide binding site of monomeric G actin, which in turn can bind DNase-1 with affinities in the nanomolar range and thus inhibits its endonuclease activity *in vitro* [22, 32, 33]. Determination of DNase-1 inhibition in cell extracts therefore enables the measurement of monomeric G actin concentration and allows the actin folding activity of TCP-1 in cells to be assessed [33]. Increase in DNase-1 inhibition was observed in caveolin transfected HuH cells, but not in mock transfected cells, upon insulin, EGF or serum stimulation. Statistical evaluation of these results by paired two-tail *t*-tests, however, revealed that only insulin can be considered as inducing a significant increase of DNase-1 inhibition ($p < 0.05$), whereas the effect of EGF remains at the lower limit of statistical significance, and the data obtained with the serum cannot at that point be considered as significant. In addition, the differences between untreated or treated non-transfected cells were not significant ($0.2 < p < 0.5$). Deoxyglucose was used as a control to block TCP-1 activity (Fig. 4). These data show that upon insulin stimulation caveolin affects TCP-1 actin folding function in intact cells. Furthermore, the absence of a significant increase in DNase-1 inhibition in mock-transfected cells upon insulin stimulation ($p < 0.5$) suggests that the mechanism by which caveolin modulates TCP-1 activity is indirect.

**Insulin-mediated phosphorylation of caveolin on Y14 induces the dissociation of the caveolin-TCP-1 complex and promotes TCP-1 folding activity.** To understand the mechanism by which caveolin modulates TCP-1 actin folding activity, we investigated how insulin-mediated cell activation increases TCP-1 actin folding in caveolin-expressing cells (Fig. 4). Insulin has been re-

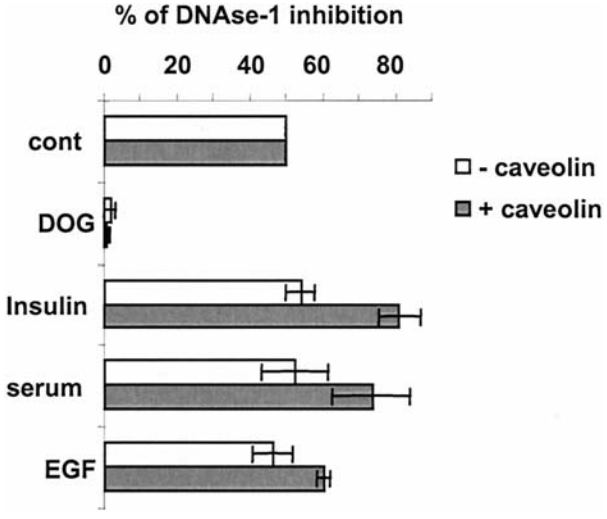


**Figure 4**. Caveolin expression modulates TCP-1 actin folding activity upon cell stimulation. HuH cells untransfected or transfected with caveolin were stimulated with insulin, EGF or serum for 5 min or pretreated with deoxyglucose for 1 h at 37 °C, lysed and the content of monomeric G actin in the whole cell lysate determined by DNase-1 inhibition assay (see Methods section). Shown are the mean and standard deviation of three independent experiments.

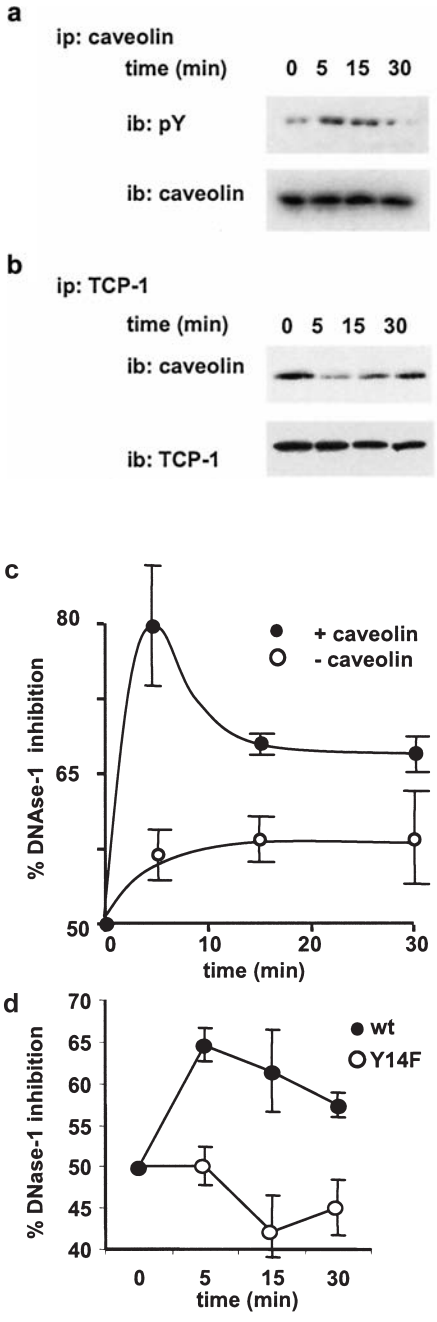


**Figure 5**. Insulin-mediated phosphorylation of caveolin induces caveolin-TCP-1 complex dissociation and activation of actin folding. Kinetic of caveolin phosphorylation (*a*) and caveolin association with TCP-1 (*b*) were examined upon addition of insulin to caveolin-transfected HuH cells. Cell lysates were immunoprecipitated with anti-caveolin- (*a*) or anti-TCP-1- (*b*) specific antibodies and the immunoprecipitates resolved by SDS-PAGE and analyzed by Western blotting using anti-phosphotyrosine (pY), anti-caveolin or anti-TCP-1$\beta$ antibodies. (*c*) Aliquots of the same HuH cell lysates were used to determine their content of monomeric G actin in by DNase-1 inhibition assay. (*d*) HuH cells transfected with either wild-type or Y14F mutant caveolin were stimulated with insulin for 5 min, lysed and the content of monomeric G actin determined by DNase-1 inhibition assay. In (*c*) and (*d*) the mean and standard deviation of three independent experiments are shown.

ported to induce caveolin phosphorylation [12] primarily at residue Y14, a reaction which is likely to result directly from the engagement of IR [10]. To determine whether the insulin-mediated phosphorylation of the N-terminal region of caveolin affects its association with TCP-1, HuH cells transfected with caveolin $\alpha$ were stimulated with insulin. After various periods of time up to 30 min, the interaction between caveolin and TCP-1 was analyzed by immunoprecipitation. Insulin treatment caused transient phosphorylation of caveolin, with an optimum reached after 5 min of stimulation (Fig. 5a). The maximal phosphorylation of caveolin correlated with a minimal binding to TCP-1 (Fig. 5b), suggesting that the phosphorylation of caveolin inhibits TCP-1 binding (Fig. 5c).

We then examined the role of caveolin phosphorylation on the actin folding activity of TCP-1. To this end, we measured the DNase-1 inhibition effect in HuH caveolin transfectants upon insulin stimulation. A maximal inhibition was detected in cell extracts after 5 min of stimulation (Fig. 5c), which corresponds to the maximum of caveolin phosphorylation (Fig. 5a). A control experiment performed with mock-transfected cells showed no significant effect of insulin treatment.

In addition, we generated a caveolin mutant in which the phosphorylation site at Y14 was replaced by a phenylalanine. Wild-type and mutant caveolin expression were comparable in both HuH and A7 caveolin-transfected cells (data not shown). Measurement of DNase-1 inhibition showed that wild-type caveolin but not the Y14F mutant induced TCP-1 folding activity in response to insulin stimulation (Fig. 5d). These results demonstrate that caveolin phosphorylation on residue Y14 is required for insulin-induced activation of TCP-1 and that caveolin is necessary to promote the TCP-1 folding activity.

**Caveolin-TCP-1 interaction acts as a competitor for caveolin-filamin interaction and thus restores insulin signaling.** To further understand the complex role of caveolin in the control of TCP-1 biological activity, we explored the possibility that caveolin might interfere with mechanisms regulating insulin-mediated cell activation. This was based on the observation that the actin-cross-linking protein filamin interacts with the IR, thereby inducing a deactivation of insulin-mediated signaling events [25]

Since caveolin-1 also binds filamin [36], we hypothesized that this interaction could interfere with the IR-filamin association, and thus restore insulin-mediated signaling and activation of TCP-1.

To test this hypothesis, A7 filamin-expressing cells were transfected with caveolin-1 $\alpha$ and the interaction between IR and filamin analyzed by co-immunoprecipitation. Indeed, expression of caveolin impaired IR-filamin interaction (Fig. 6a) by 45–50% as determined by the quantitation of the relative intensity of ECL signals (see Methods section). The impact of the competition between filamin

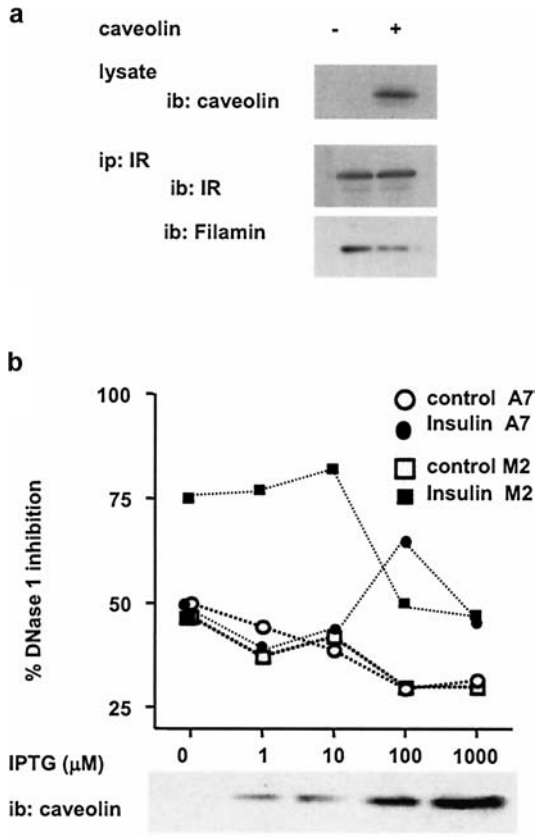


**Figure 6.** Role of caveolin in insulin-induced activation of TCP-1. (*a*) Interaction of the IR with filamin in A7 cells transfected or not with caveolin. The IR was immunoprecipitated with specific antibodies and the immunoprecipitates resolved by SDS-PAGE followed by Western blotting using anti-filamin- or anti-IR-specific antibodies. Equal amounts of protein cell lysates were examined by immunoblotting using anti-caveolin-specific antibodies (top panels). (*b*) Insulin-mediated folding of G actin in filamin-positive (A7) and filamin-negative (M2) cells expressing increasing concentrations of caveolin. Shown is one representative out of three independent experiments (*a* and *b*) at optimal time of DNase-1 inhibition, *i.e.*, 10 min after insulin stimulation (*b*).

binding to caveolin or the IR on TCP-1 activation was then assessed using A7 and the derived filamin-deficient M2 cell lines. Both cell types were transfected with a plasmid encoding caveolin-1 $\alpha$ under the control of an IPTG-inducible promoter, and the activity of TCP-1 was determined by the measure of DNase-1 inhibition following insulin stimulation.

In A7 cells, in the absence of caveolin expression, no change in DNase-1 inhibition was detected upon 10 min of insulin treatment (Fig. 6b). By contrast, following induction of caveolin expression with graduated doses of IPTG, a significant increase in DNase-1 inhibition was observed at 100 μM IPTG, corresponding probably to the optimal level of caveolin-1 expression inducing the strongest effect. Lower or higher expression levels of caveolin failed to induce any detectable change in DNase-1 inhibition upon insulin stimulation (Fig. 6b). Thus in A7 cells, like in HuH cells, caveolin regulates its folding activity *via* its association with TCP-1 (Fig. 5c).

By contrast to A7 cells, when M2 filamin-deficient cells were used, insulin induced a stronger DNase-1 inhibition in the absence of caveolin expression (Fig. 6b). This confirms the selective impairment in insulin signaling by filamin [25]. Importantly, in both A7 and M2 cells expressing levels of caveolin higher than that obtained with 100 μM and 1000 μM IPTG, respectively, insulin stimulation failed to induce DNase-1 inhibition, indicating that caveolin can inhibit TCP-1 activation (Fig. 6b). Further evidence for the direct inhibitory effect of caveolin on TCP-1 actin folding function was provided by the experiment using the caveolin mutant Y14F, which showed impaired activation of TCP-1 upon insulin stimulation (Fig. 5d). This effect was independent of the level of caveolin expression (data not shown). In addition, at low expression levels (up to 10 μM IPTG), caveolin can restore insulin-mediated activation of TCP-1 in A7 cells (Fig. 6a)

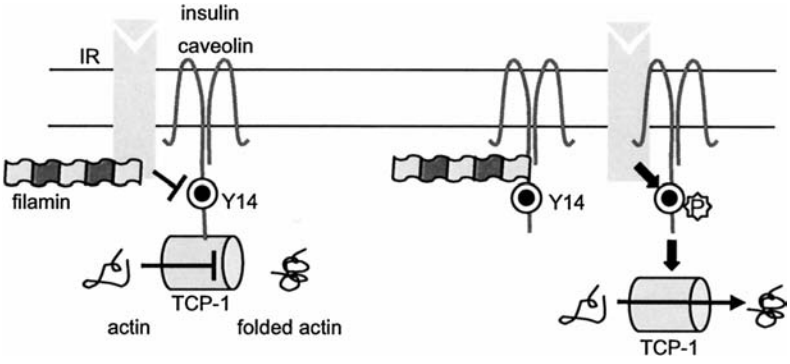


**Figure 7**. Mechanism of caveolin regulation of TCP-1 activity. Filamin, by interacting with the IR, inhibits insulin-mediated signaling. At low expression level, caveolin does not compete efficiently for this interaction but, by associating with TCP-1, inhibits its actin folding function (left panel). At higher expression level, caveolin sequesters filamin from the IR and allows insulin-mediated phosphorylation of caveolin at Y14 and the release of the functionally immobilized TCP-1 (right panel).

by competing for the IR-filamin interaction (Fig. 6b). Taken together these results indicate that caveolin-1 can function as a direct inhibitor of TCP-1 folding activity and as an indirect activator of TCP-1 by competing for the filamin-IR interaction.

## Discussion

In the present study, we have shown that caveolin binds the chaperone complex TCP-1 and thus contributes to the regulation of its protein folding function. The N-terminal region of caveolin-1 $\alpha$ is crucial for that interaction as evidenced by the specific binding of TCP-1 to a 32-amino acid synthetic peptide corresponding to the N terminus of the protein. Moreover, TCP-1 can be co-immunoprecipitated with native caveolin-1 $\alpha$, whereas only a weak interaction was observed between the chaperone complex and caveolin-1 $\beta$, an isoform of caveolin-1 that lacks the 32 N-terminal residues.

Several other proteins were consistently found in association with the caveolin-1 N-terminal peptide, among which ATP synthase and actin could be identified. The presence of actin is consistent with its specific binding to TCP-1 under the mild dispersing conditions used in our experiments. Indeed, the actin-TCP-1 interaction is highly resistant to the disruption in mixed micelles of ionic and non-ionic detergents that cause complete disruption of the TCP-1 hexadecamer into its constituent subunits [37]. 2D electrophoresis revealed a number of spots corresponding to the various subunits of TCP-1 [38, 39], among which the subunits $\beta$ and $\theta$ were in a sufficient amount to allow their identification by MS. Our data show that TCP-1 interacts with the N-terminal segment of caveolin, but due to the fact that under the experimental conditions used, TCP-1 dissociates partially into its constituent subunits, it remains difficult to evaluate the proportion of TCP-1 complexes associated with caveolin. Our data are consistent with a quantitative proteomic analysis demonstrating that both caveolin and TCP-1 partitioned in glycolipid-enriched membrane microdomains [40]. Considering that the cytosolic chaperone complex TCP-1 is expected to partition in the detergent soluble fractions, its localization in rafts might result from its association with caveolae cytosolic organelles.

Using a DNase I-inhibition assay, which measures the amount of folded actin [32, 33], we provide evidence that caveolin exerts two competing effects on TCP-1 actin folding function. Caveolin, by binding directly to TCP-1, inhibits the chaperone actin folding function as observed in filamin-deficient cells expressing large amounts of caveolin (Fig. 6b). Further evidence for a direct inhibition of TCP-1 by caveolin were obtained by the observation that the Y14F caveolin mutant does not induce TCP-1 activation nor dissociation from TCP-1 upon insulin stimulation (Fig. 5). These data demonstrate that the specific phosphorylation of this residue controls both the caveolin-TCP-1 interaction and the chaperone folding function (Fig. 7).

By preventing the actin cross-linking protein filamin to bind to the IR, caveolin restores insulin-mediated signaling and activates TCP-1 actin folding function (Fig. 6b). The fragment of filamin spanning repeats 22, 23 and the hinge region are involved in the interaction with both caveolin and the IR [25, 36]. Caveolin interacts with TCP-1 mainly through its first 32 N-terminal residues and with filamin *via* residues 32–101 (Figs 1 and 2). In addition, a possible negative influence of the first 32 N-terminal residues on the strength of this later interaction has been described [25]. The filamin-caveolin complex sequesters filamin from the IR, and thus prevents the phosphorylation of caveolin associated with filamin (Fig. 6, right panel). This complex, by sequestering filamin from the IR, restores insulin signaling through the IR-caveolin-TCP-1 complex. Consequently, insulin mediates phosphorylation of caveolin at Y14, which in turn induces the dissociation of caveolin from TCP-1 and the activation of the chaperone (Fig. 6, right panel). Thus, low expression of caveolin in filamin-expressing cells may not be sufficient to compete for filamin-IR interaction (Fig. 6. from left to right panel). In contrast, in filamin-deficient cells, such a low level of caveolin is sufficient to inhibit TCP-1 activity, and to override insulin-mediated activation of TCP-1. Consequently, at a high level of expression of caveolin, the formation of the complex with TCP-1 prevails over its insulin-mediated dissociation. This is most likely due to the fact that insulin-mediated phosphorylation of caveolin is transient (Fig. 3a).

Moreover, by forming such a complex, the chaperone TCP-1 could modulate the conformation and the functions of caveolin. In fact, a key role of caveolin is to act as a negative regulator of signaling molecules, a function that was initially attributed to the scaffolding domain [5, 9] and more recently to a specific active conformation of the protein [41].

Our data show that a functional consequence of caveolin-TCP-1 interaction is the regulation of the insulin-mediated mitogenic pathway. Similar effects of TCP-1 actin folding function was obtained upon EGF or serum treatment (Fig. 4), indicating that mediators of caveolin phosphorylation release the functionally sequestered chaperone. However, it remains to be determined whether the mechanism identified for insulin stimulation is similar for EGF and serum treatment, and represents a more general mechanism of caveolin regulatory function.

An additional important function of caveolin-TCP-1 interaction may be to regulate the interaction of caveolae with the cortical actin cytoskeleton. Caveolae tend to collect in the actin-rich region of the cell membrane and contain the actin and caveolin binding protein filamin. There-

fore, by regulating actin folding function of TCP-1, caveolin may control the vesicular traffic of caveolae between the cell surface and intracellular organelles.

*Acknowledgement.* We are grateful to Drs T. Stossel and Y. Ohta for providing the M2 and A7 cell lines (Brigham and Women's Hospital, Harvard Medical School, Boston, USA). We thank Léonard Bagnoud for technical assistance, Dr. Slavica Masina (Institute of Biochemistry, Epalinges, Switzerland), Dr. Olivier Michielin (ISREC, Epalinges, Switzerland) and Prof. Andreas Conzelman (University of Fribourg, Switzerland) for careful reading of the manuscript. This study was supported by the Swiss National Science Foundation (SNF 31-61960) and the Giorgi-Cavalieri Foundation.

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