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β2-Adaptin is constitutively de-phosphorylated by serine/threonine protein phosphatase PP2A and phosphorylated by a staurosporine-sensitive kinase

Jens Peter H. Lauritsen, Charlotte Menné, Jesper Kastrup, Jes Dietrich, Niels Ødum, Carsten Geisler *

Institute of Medical Microbiology and Immunology, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark

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Abstract

Clathrin-mediated endocytosis includes cycles of assembly and disassembly of the clathrin-coated vesicle constituents. How these cycles are regulated is still not fully known but previous studies have indicated that phosphorylation of coat subunits may play a role. Here we describe that β2-adaptin undergoes cycles of phosphorylation/de-phosphorylation in intact cells. Thus, β2-adaptin was constitutively de-phosphorylated by serine/threonine protein phosphatase 2A and phosphorylated by a staurosporine-sensitive kinase in vivo. Confocal laser scanning microscopy demonstrated that phosphorylated AP2 complexes were found more evenly distributed at the plasma membrane compared to non-phosphorylated AP2 complexes which were found in aggregates. Finally, we found that phosphorylation of β2-adaptin correlated with inhibition of clathrin-mediated endocytosis. Our results support the hypothesis that phosphorylation/de-phosphorylation of coat proteins plays a regulatory role in the assembly/disassembly cycle of clathrin-coated vesicles.

Keywords: Adaptin; Adaptor; Phosphorylation; Phosphatase; Kinase; Endocytosis

1. Introduction

Clathrin-mediated endocytosis is essential in endocytosis of plasma membrane receptors and their ligands. The major coat constituents of clathrin-coated vesicles are clathrin and adaptor protein (AP) complexes [1–3]. Clathrin consists of three heavy chains each bound to one of two light chains, a or b. To date, four AP complexes known as AP1, AP2, AP3, and the newly discovered AP4 have been described [4–6]. Whereas AP1, AP3, and possibly AP4 are involved in intracellular transport events, AP2 is involved in clathrin-mediated endocytosis from the plasma membrane. All four AP complexes consist of two large subunits (γ, α, δ, or ε and β1, β2, β3, or β4, ∼90–140 kDa) called adaptins, one medium subunit (μ1, μ2, μ3, or μ4, ∼50 kDa) and one small subunit (σ1, σ2, σ3, or σ4, ∼20 kDa). The β1-

Abbreviations: AP, adaptor protein; PP, serine/threonine protein phosphatase; PE, phycoerythrin; PDB, phorbol-12,13-dibutyrate; PKC, protein kinase C; PKA, protein kinase A; MFI, mean fluorescence intensity; TCR, T cell receptor; FCS, fetal calf serum; mAb, monoclonal antibody

* Corresponding author. Fax: +45-3532-7881;
E-mail: cgtcr@biobase.dk

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and β2-adaptins interact with clathrin through a clathrin box sequence and induce clathrin to form cages [7–9]. The μ2-chain binds tyrosine-based receptor-sorting motifs in the form Yppø where Y is tyrosine, p is a polar residue, and ø is a residue with a bulky hydrophobic side chain [10]. Leucine-based receptor-sorting motifs are recognized by either the β2- or the μ2-chains [11–13]. The α-adaptin has been reported to interact with accessory factors involved in clathrin coat assembly such as dynamin, amphiphysin, epsin and Eps15 [14–18]. The exact functions of the γ-, δ-, ε-adaptins and the σ-subunits still have to be determined.

Clathrin-mediated endocytosis includes cycles of assembly and disassembly of the clathrin-coated vesicle constituents. Formation of a clathrin-coated vesicle is initiated by recruitment of AP2 complexes from the cytosol to the plasma membrane. Additional coat constituents are recruited and assembled stepwise, incrementally deforming the attached segment of the membrane in process. The vesicle is released encased in the clathrin coat, which is then discarded to allow the vesicle to fuse with the target compartment [3,19]. The uncoating ATPase, hsc70, probably mediates the release of clathrin from the vesicle, however, hsc70 does not release AP2 [20–22]. Factors required for release of AP2 from coated vesicles remain to be identified.

Interestingly, clathrin [23,24], the AP2 complex proteins (α, β_2, μ_2, σ_2) [25], dynamin 1, and amphiphysin [26–28] all have the ability to be phosphorylated on either serine or threonine. Thus, phosphorylation/de-phosphorylation of coat proteins might play a regulatory role in the assembly/disassembly cycles of clathrin-coated vesicles. Accordingly, results from previous studies have suggested that phosphorylation of coat proteins may play a role in controlling the assembly state [25,29–31]. Thus, it was found that phosphorylated AP2 complexes are unable to bind to clathrin cages in vitro and that phosphorylated AP2 complexes mainly are localized to the cytosol [25]. Likewise, interaction of amphiphysin 1 with the major coat constituents in vitro is inhibited by phosphorylation of amphiphysin 1 [31]. If phosphorylation/de-phosphorylation of coat proteins plays an important role in assembly/disassembly of clathrin-coated vesicles it should be expected that a constitutive phosphorylation and de-phosphorylation of coat proteins takes place in intact cells. However, the kinases and phosphatases responsible for phosphorylation/de-phosphorylation of coat proteins in vivo remain to be identified.

The aim of this study was to determine whether β-adaptin undergoes cycles of phosphorylation/de-phosphorylation in intact cells and if so, to further characterize potential kinases and phosphatases involved in this process. We found that β-adaptin was constitutively de-phosphorylated by serine/threonine protein phosphatase (PP) 2A and phosphorylated by a staurosporine-sensitive kinase in vivo. Confocal laser scanning microscopy demonstrated that phosphorylated AP2 complexes were found at the plasma membrane. However, compared to non-phosphorylated AP2 that were found in aggregates, phosphorylated AP2 complexes were more evenly distributed. Finally, we found that phosphorylation of β2-adaptin correlated with inhibition of clathrin-mediated endocytosis of receptors containing either leucine- or tyrosine-based receptor-sorting motifs.

2. Materials and methods

2.1. Cells, antibodies, and chemicals

The human Jurkat T cell line J76 and the transfectant CD4/3-SA, JGN transfected with a chimeric CD4/CD3γ molecule as previously described [32,33], were cultured in RPMI 1640 medium supplemented with penicillin 2×10^5 U/l (Leo Pharmaceutical Products, Ballerup, Denmark), streptomycin 50 mg/l (Merck, Darmstadt, Germany), and 10% (vol/vol) fetal calf serum (FCS) (Life Technologies, Paisley, UK) at 37°C in 5% CO_2. The anti-β-adaptin monoclonal antibody (mAb) 100/1 recognizing β1- and β2-adaptin was obtained from Sigma Chemicals (St. Louis, CA, USA). Anti-α-adaptin was from Oncogene Research Products (Cambridge, MA, USA). Phycoerythrin (PE)-conjugated mouse anti-human CD3ε mAb (UCHT1) and peroxidase-conjugated rabbit anti-mouse Ig were obtained from Dakopatts (Glostrup, Denmark). The anti-TCR mAb F101.01 was produced in our own laboratory [34]. PE-conjugated anti-mouse CD4 was from Pharmingen (San Diego, CA, USA). PE-conjugated $F(ab)_2$ fragments of goat anti-mouse Ig H+L was obtained from Jack-

son Immunoresearch (West Grove, PA, USA). The serine/threonine protein phosphatase inhibitors tautomycin and okadaic acid were from Calbiochem (La Jolla, CA, USA) and calyculin A was from Biomol (Plymouth Meeting, PA, USA). Calf intestinal alkaline phosphatase (CIAP) was from New England Biolabs (Beverly, MA, USA). Purified PP1 from rabbit skeletal muscle and purified PP2A from human red blood cells were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The phorbol ester phorbol-12,13-dibutyrate (PDB) was from Sigma Chemicals. Transferrin–fluorescein was from Boehringer-Mannheim (Mannheim, Germany) and deferoxamine was obtained from Ciba-Geigy AG (Basel, Switzerland). Fluorescein–dextran, 70 000 MW, was from Molecular Probes (Leiden, The Netherlands). The kinase inhibitors staurosporine, KT5720, KT5823, bisindolylmaleimide I, KN93, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were all from Calbiochem. The protein kinase A (PKA) activators 8-CPT-cAMP or sp-cAMPS or the PKA inhibitor rp-cAMPS were from Biomol.

2.2. Immunoprecipitation and Western blot

For immunoprecipitation cells (1×10^7) were adjusted to 1×10^6 cells per ml of medium (RPMI 1640+10% FCS) and incubated for 1 h at 37°C with or without inhibitor. Subsequently the cells were washed three times in ice-cold PBS and lysed in 0.5 ml lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 10 mM EDTA, 10 mM NaF, 1 mM aprotinin, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 100 nM calyculin) for 20 min on ice. The lysates were precleared three times with protein A-Sepharose (PA) beads (Amersham Pharmacia Biotech, Sweden), incubated with 20 μl anti-α-adaptin mAb for 1.5 h and subsequently incubated with PA for another 1.5 h. The PA beads were washed three times in lysis buffer, before elution of the proteins from the beads by boiling for 5 min in sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol, 2% mercaptoethanol). Samples were electrophoresed in 10% polyacrylamide gels and subsequently analyzed by Western blot as described below.

For Western blots, cells were adjusted to 1×10^6 cells per ml of medium (RPMI 1640+10% FCS) and incubated at 37°C for 1 h or the time indicated with the various inhibitors. The cells were subsequently lysed in lysis buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40) for 20 min on ice. The lysates were centrifuged for 10 min at $4500\times g$, 4°C and subsequently boiled for 5 min in sample buffer. Following SDS–PAGE, the proteins were transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech) by semi-dry blotting. Membranes were blocked in 1% BSA, 4% non-fat milk in PBS for 1 h and subsequently incubated with anti-β-adaptin mAb (1:500 dilution) overnight. Detection of anti-β-adaptin was performed using a 1:2000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse Ig. Bound horseradish peroxidase-conjugated rabbit anti-mouse Ig was visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

2.3. Confocal laser scanning microscopy

Cells were washed in PBS and fixed for 10 min with 2% paraformaldehyde/PBS. The cells were permeabilized for 10 min at room temperature with washing buffer (HEPES-buffered PBS, containing 0.1% saponin) and stained with anti-α-adaptin mAb at room temperature for 20 min. Cells were washed three times in washing buffer and stained with Cy5-conjugated $F(ab)_2$ fragments of donkey anti-mouse IgG H+L (Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 20 min. Confocal microscopy was performed on a Zeiss LSM510 connected to a Zeiss Axiovert 100 M microscope (Carl Zeiss Ltd., Jena, Germany). Cy5 fluorescence was detected using long pass filter LP 650.

2.4. TCR, transferrin receptor, and CD4/3-SA endocytosis

For TCR endocytosis, cells were adjusted to 1×10^6 cells per ml of medium (RPMI 1640+10% FCS) and pre-incubated at 37°C for 1 h with the indicated phosphatase inhibitors. PDB or the TCR ligand mAb F101.01 was added and the cells were incubated for one additional hour at 37°C. Subsequently, the cells were transferred to ice-cold PBS containing 2% FCS and 0.1% NaN_3 and washed

twice. Cells treated with PDB were stained directly with PE-conjugated anti-CDε and analyzed using a FACScalibur (Becton Dickinson, Mountain View, CA, USA). Cells stimulated with F101.01 were stained with F101.01 followed by PE-conjugated $F(ab)_2$ fragments of goat anti-mouse Ig H+L. Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent antibody binding (MFI of cells treated with PDB/F101.01 and phosphatase inhibitor)/(MFI of cells treated only with phosphatase inhibitor)$\times$100%.

For transferrin endocytosis, cells were adjusted to 1×10^6 cells per ml of medium (RPMI 1640+10% FCS) and pre-incubated at 37°C for 1 h with the indicated phosphatase inhibitors. The cells were subsequently incubated at 37°C with fluorescein–transferrin (4 μg/ml). At the indicated times aliquots of the cell suspension were washed in ice-cold PBS containing 2% FCS and 0.1% NaN_3 and incubated for 20 min at 4°C with 50 mM citric acid, pH 5.0, 100 mM NaCl, 100 μM deferoxamine and 0.2% BSA to strip of cell surface bound transferrin. After 20 min the cells were washed once with ice-cold PBS and incubated for 15 min at 4°C with PBS containing 100 mM deferoxamine and 0.2% BSA. Subsequently, the cells were analyzed on a FACScalibur. MFI was recorded and used in the calculation of percent endocytosed transferrin–fluorescein (MFI of phosphatase inhibitor treated cells)/(MFI of untreated cells at 30 min)$\times$100%.

For CD4/3-SA endocytosis, cells were incubated at a cell density of 1×10^6 cells per ml medium at 37°C or 4°C with PE-conjugated anti-CD4 mAb. At the time indicated aliquots of cell suspension were washed in ice-cold PBS containing 2% FCS and 0.1% NaN_3 and immediately treated with 300 μl 0.5 M NaCl, 0.5 M acetic acid, pH 2.2 for 10 s. The acid resistant fluorescence of the cells (representing internalized anti-CD4 mAb) was measured in the FACScalibur. The percentage of internalized anti-CD4 mAb to cell surface bound anti-CD4 mAb was subsequently calculated using the equation: ((HAR−CAR)/CT)$\times$100%, where HAR is the MFI of acid treated cells incubated at 37°C, CAR is the MFI of acid treated cells incubated at 4°C, and CT is the MFI of untreated cells incubated at 4°C.

3. Results

3.1. β2-Adaptin is constitutively de-phosphorylated by PP2A

It has been demonstrated that α- and β2-adaptin of AP2 and β1-adaptin of AP1 are phosphorylated on serines in vivo [25]. The phosphorylated adaptors were mainly found in the cytosol and their binding to clathrin cages was impaired in vitro. These observations indicated that adaptor subunits must be dephosphorylated to participate in clathrin-mediated vesicular transport. To determine whether the phosphatases PP1 and/or PP2A were involved in β2-adaptin de-phosphorylation, Jurkat cells were treated with the phosphatase inhibitor calyculin A, a potent inhibitor of PP1 and PP2A [35]. Cells were incubated with 60 nM calyculin A for 1 h and subsequently lysed. β2-Adaptin was co-precipitated from the lysate using the α-adaptin mAb and the precipitates were run on SDS–PAGE, transferred to membranes and immunoblotted with anti-β-adaptin mAb 100/1. The α-adaptin mAb clearly co-precipitated β2-adaptin (Fig. 1A, lane 1 versus lane 2). Interestingly, treatment with calyculin A induced a mobility shift of β2-adaptin, which could be caused by β2-adaptin phosphorylation (Fig. 1A). Parallel analyses of whole-cell lysates and α-adaptin immunoprecipitates gave identical results, which allowed us directly to analyze whole-cell lysates in the following experiments. To determine the dose-dependency of the calyculin A-mediated mobility shift of β-adaptin, cells were incubated with different concentrations of calyculin A for 1 h and subsequently lysed. The mobility shift of β-adaptin was observed at 40 nM calyculin A and was almost complete at 120 nM (Fig. 1B). To investigate whether the mobility shift actually was caused by phosphorylation of β-adaptin, lysates from cells treated with 120 nM calyculin A were treated with alkaline phosphatase. As shown in Fig. 1B, alkaline phosphatase treatment of the lysate completely reversed the mobility shift, demonstrating that the calyculin A induced mobility shift of β-adaptin was caused by phosphorylation of β-adaptin. To determine the kinetics of β-adaptin phosphorylation, cells were incubated with 60 nM calyculin A for different

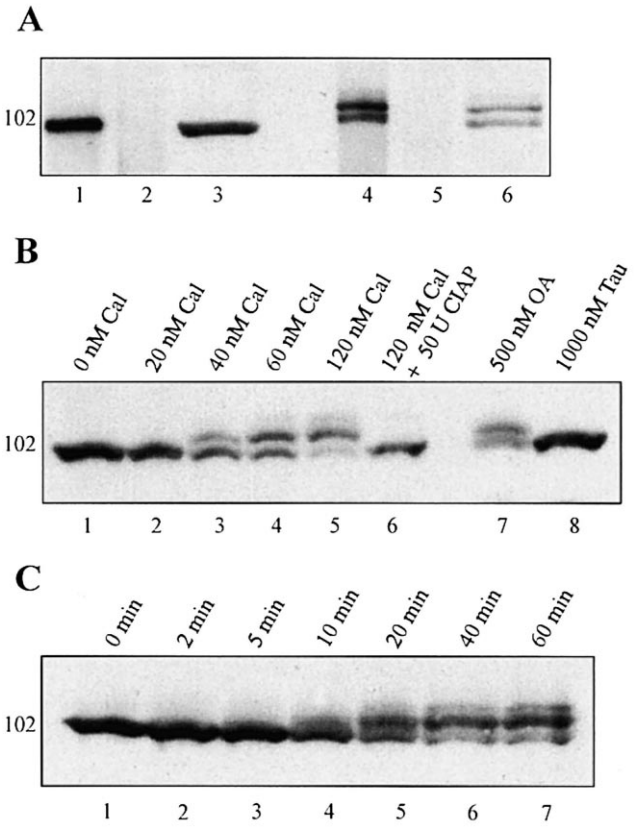

Fig. 1. β2-Adaptin is constitutively de-phosphorylated by PP2A. (A) Cells were incubated without (lanes 1–3) or with (lanes 4–6) 60 nM of calyculin A for 1 h. The cells were subsequently lysed and the lysates were either precipitated with anti-α-adaptin mAb (lanes 1 and 4), with irrelevant mAb (lanes 2 and 5), or left untreated (lanes 3 and 6). The precipitated material and whole-cell lysates were subsequently run on SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with the anti-β-adaptin mAb 100/1. (B) Cells were incubated with increasing concentrations of calyculin A (Cal, lanes 1–6), okadaic acid (OA, lane 7) or tautomycin (Tau, lane 8) for 1 h. The cells were subsequently lysed and the lysates were either treated with calf intestinal alkaline phosphatase (CIAP, lane 6) for 2 h at 37°C or kept on ice (lanes 1–5, 7, and 8). The lysates were run on SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with the anti-β-adaptin mAb 100/1. (C) Cells were incubated with calyculin A (60 nM) for the periods indicated. The cells were subsequently lysed and the lysates analyzed as described above.

periods. Following calyculin A treatment, the cells were lysed and analyzed as described above. β-Adaptin phosphorylation was observed within 10 min and the mobility shift was almost complete after 40 min (Fig. 1C).

Calyculin A does not significantly discriminate between PP1 and PP2A [35]. To determine the main phosphatase involved in β-adaptin de-phosphorylation, cells were treated with okadaic acid, a potent PP2A inhibitor, or tautomycin that preferably inhibits PP1 [35,36]. Okadaic acid induced the same kind of mobility shift of β-adaptin as calyculin A, whereas tautomycin did not indicating that PP2A and not PP1 de-phosphorylated β-adaptin (Fig. 1B, lanes 7 and 8). This was further supported by the observation that treatment of phosphorylated β-adaptin in vitro with purified PP2A completely de-phosphorylated β-adaptin from as well whole-cell lysate as well purified β2-adaptin. In contrast, treatment with purified PP1 only partially de-phosphorylated β-adaptin (Fig. 2). Finally, addition of calyculin A (120 nM) to the lysis buffer of untreated cells did not cause the appearance of phosphorylated β-adaptin, demonstrating that constitutively phosphorylation of β-adaptin took place in intact cells and not in the cell lysate (data not shown).

Taken together, these results demonstrated that β2-adaptin is constitutively de-phosphorylated in vivo by a phosphatase belonging to the PP2A family.

3.2. *β-Adaptin is phosphorylated by a staurosporine-sensitive kinase*

The observation that β-adaptin was constitutively de-phosphorylated implied that β-adaptin also had to be constitutively phosphorylated. In an attempt to identify the kinase(s) responsible for phosphoryla-

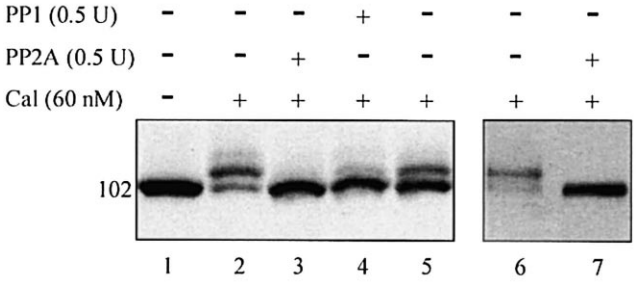

Fig. 2. β2-Adaptin is a substrate for PP2A. Cells were treated with (lanes 2–7) or without (lane 1) calyculin A (Cal, 60 nM) for 1 h. The cells were subsequently lysed. Whole-cell lysates (lanes 1–5) and anti-α-adaptin precipitates (lanes 6 and 7) were treated with purified PP2A (0.5 units, lanes 3 and 7), purified PP1 (0.5 units, lane 4), left untreated at 37°C (lanes 5 and 6), or untreated on ice (lanes 1 and 2) for 2 h. The lysates were thereafter analyzed as described above.

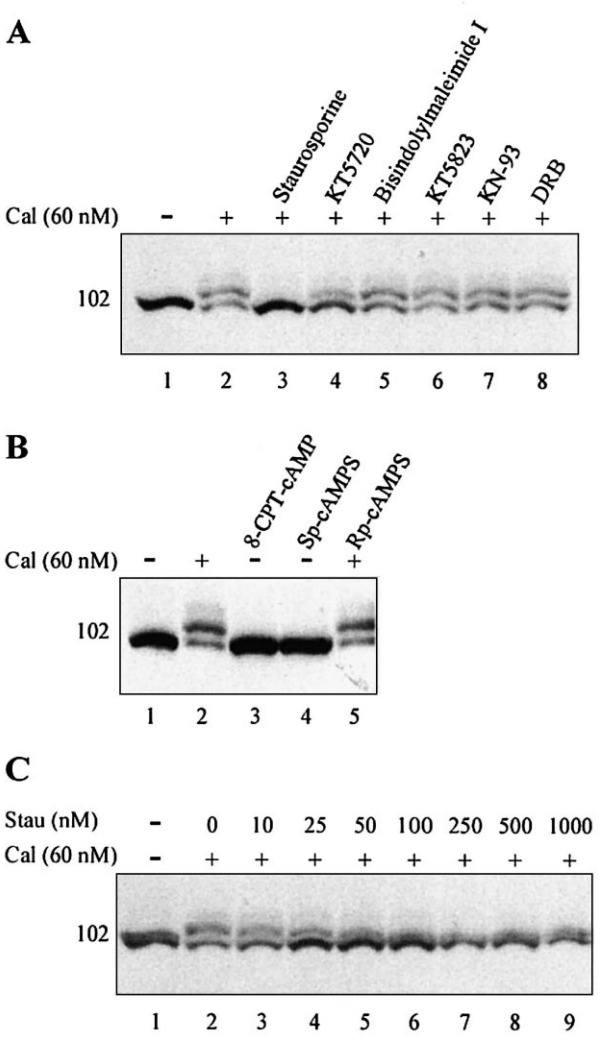

Fig. 3. β-Adaptin is phosphorylated by a staurosporine-sensitive kinase. (A) Cells were pre-incubated for 1 h with the broad kinase inhibitor staurosporine (1000 nM, lane 3), the PKA inhibitor KT5720 (1000 nM, lane 4), the PKC inhibitor bisindolylmaleimide I (1000 nM, lane 5), the protein kinase G inhibitor KT5823 (1000 nM, lane 6), the calmodulin kinase II inhibitor KN93 (10 μM, lane 7) and the casein kinase II inhibitor DRB (100 μM, lane 8) before co-incubation with (lanes 2–8) or without (lane 1) calyculin A (60 nM) for an additional 1 h. The cells were subsequently lysed and the lysates run on SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with the anti-β-adaptin mAb 100/1. (B) Cells were pre-incubated for 1 h with the PKA activators 8-CPT-cAMP (500 μM, lane 3) or sp-cAMPS (100 μM, lane 4) or the PKA inhibitor rp-cAMPS (200 μM, lane 5) before co-incubation with (lanes 2 and 5) or without (lanes 1, 3, and 4) calyculin A (60 nM) for an additional 1 h. The cells were subsequently lysed and the lysates analyzed as described above. (C) Cells were pre-incubated for 1 h with increasing concentrations of staurosporine before co-incubation with (lanes 2–9) or without (lane 1) calyculin A (60 nM) for an additional 1 h. The cells were subsequently lysed and the lysates analyzed as described above.
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tion of β-adaptin, cells were treated with different kinase inhibitors prior to incubation with calyculin A. The cells were lysed and the lysates run on SDS–PAGE, transferred to membranes and immunoblotted with anti-β-adaptin mAb. No or reduced levels of β-adaptin phosphorylation should be observed following calyculin A treatment if the kinase inhibitor tested inhibited the β-adaptin kinase. The broad-spectrum kinase inhibitor staurosporine almost completely abolished β-adaptin phosphorylation (Fig. 3A). Furthermore, the protein kinase A (PKA) inhibitor KT5720 reduced phosphorylation of β-adaptin. None of the other kinase inhibitors affected β-adaptin phosphorylation. Whether PKA actually was involved in β-adaptin phosphorylation was next analyzed using specific inhibitors or activators of PKA. If PKA was involved an increase in β-adaptin phosphorylation would be expected in cells treated with PKA activators and a reduction in β-adaptin phosphorylation should be observed in cells treated with PKA inhibitors. However, neither the PKA activators 8-CPT-cAMP and sp-cAMPS nor the PKA inhibitor rp-cAMPS affected β-adaptin phosphorylation (Fig. 3B) indicating that PKA was not involved in β-adaptin phosphorylation. To further characterize the staurosporine-sensitive β-adaptin kinase, cells were pre-incubated with various concentrations of staurosporine for 1 h followed by co-incubation with calyculin A (60 nM). A reduction in β-adaptin phosphorylation was observed in cells treated with 25 nM staurosporine and β-adaptin phosphorylation was almost totally abolished in cells treated with 100 nM staurosporine (Fig. 3C). Taken together, these results demonstrated that β-adaptin is constitutively phosphorylated by a staurosporine-sensitive kinase.

3.3. *Phosphorylation influences the distribution of AP2 complexes at the plasma membrane*

In a previous study on bovine kidney cells, phosphorylated AP2 complexes predominated in the cy-

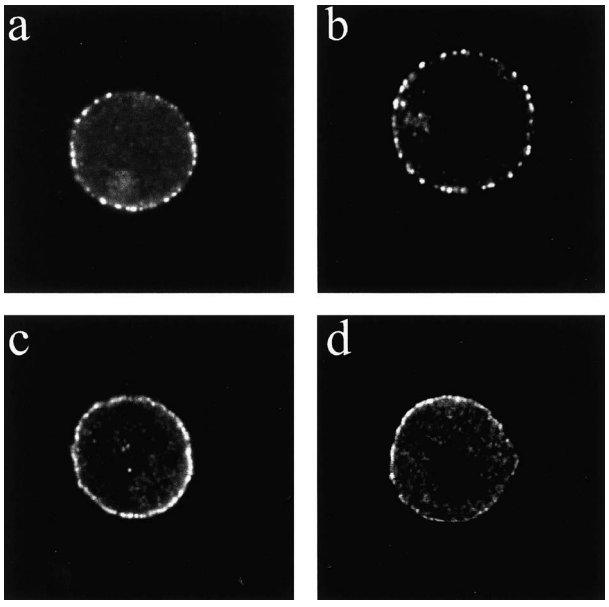

Fig. 4. Phosphorylation influences the distribution of AP2 complexes at the plasma membrane. Cells were incubated without (a and b) or with (c and d) 120 nM of calyculin A for 1 h. The cells were subsequently fixed, permeabilized, stained with anti-α-adaptin mAb and analyzed by confocal laser scanning microscopy.

tosol in comparison to AP2 complexes associated with the plasma membrane [25]. To determine the intracellular distribution of AP2 complexes containing phosphorylated β2-adaptin in human T cells, cells were treated with 120 nM calyculin A previously shown to induce phosphorylation of the vast majority of β-adaptin molecules (Fig. 1B, lane 5). The cells were subsequently fixed, permeabilized, stained with anti-α-adaptin mAb and analyzed by confocal laser scanning microscopy. The staining pattern of calyculin A treated cells differed from untreated cells (Fig. 4). Thus, whereas AP2 complexes in untreated cells were mainly found in aggregates at the plasma membrane, AP2 complexes in calyculin A treated cells were more evenly distributed at the plasma membrane. However, there did not seem to be a significant redistribution of AP2 complexes from the plasma membrane to the cytosol as a result of the induced phosphorylation.

3.4. *Phosphorylation-induced T cell receptor endocytosis is blocked by PP2A inhibitors*

Previous studies have described an impaired interaction between proteins involved in clathrin-mediated endocytosis following their phosphorylation [25,31]. These observations suggest that clathrin-mediated endocytosis probably is inhibited during circumstances that promote phosphorylation. First, we used the T cell receptor (TCR) as a model to study the effect of phosphatase inhibitors on clathrin-mediated endocytosis. TCR endocytosis can be induced by two different pathways dependent on either tyrosine or serine phosphorylation of the TCR components [37]. Thus, the TCR becomes tyrosine phosphorylated and subsequently endocytosed following ligand binding. Likewise, the TCR is endocytosed following protein kinase C (PKC)-mediated phosphorylation of serine 126 in the di-leucine-based (Ser-Asp-Lys-Gln-Thr-Leu-Leu) receptor-sorting motif of CD3γ [38]. In both pathways, TCR endocytosis takes place via clathrin-coated pits and vesicles [32,38–40]. To determine the effect of the phosphatase inhibitors on TCR endocytosis, cells were pre-incubated with increasing concentrations of either calyculin A, okadaic acid or tautomycin followed by incubation with a fixed concentration of either the TCR ligand mAb F101.01 or the PKC activator PDB. Calyculin A and okadaic acid inhibited both ligand- and PKC-mediated TCR endocytosis, whereas tautomycin showed only a minor effect at the highest concentrations tested (Fig. 5A–C). Interestingly, the concentrations of calyculin A and okadaic acid required to inhibit TCR endocytosis were coincident with the concentrations required to inhibit dephosphorylation of β-adaptin. To analyze whether higher concentrations of PDB could overrule the inhibitory effect of calyculin A and okadaic acid on TCR endocytosis the following experiments were performed. Cells were pre-incubated with a fixed concentration of calyculin A (60 nM) or okadaic acid (500 nM) shown to inhibit TCR endocytosis or tautomycin (1000 nM) followed by incubation with increasing concentrations of PDB. Even the highest concentrations of PDB did not rescue PKC-mediated TCR endocytosis in the presence of calyculin A or okadaic acid (Fig. 5D). Again, tautomycin showed no inhibitory effect on TCR internalization. Similar results were obtained with ligand-induced TCR endocytosis (data not shown).

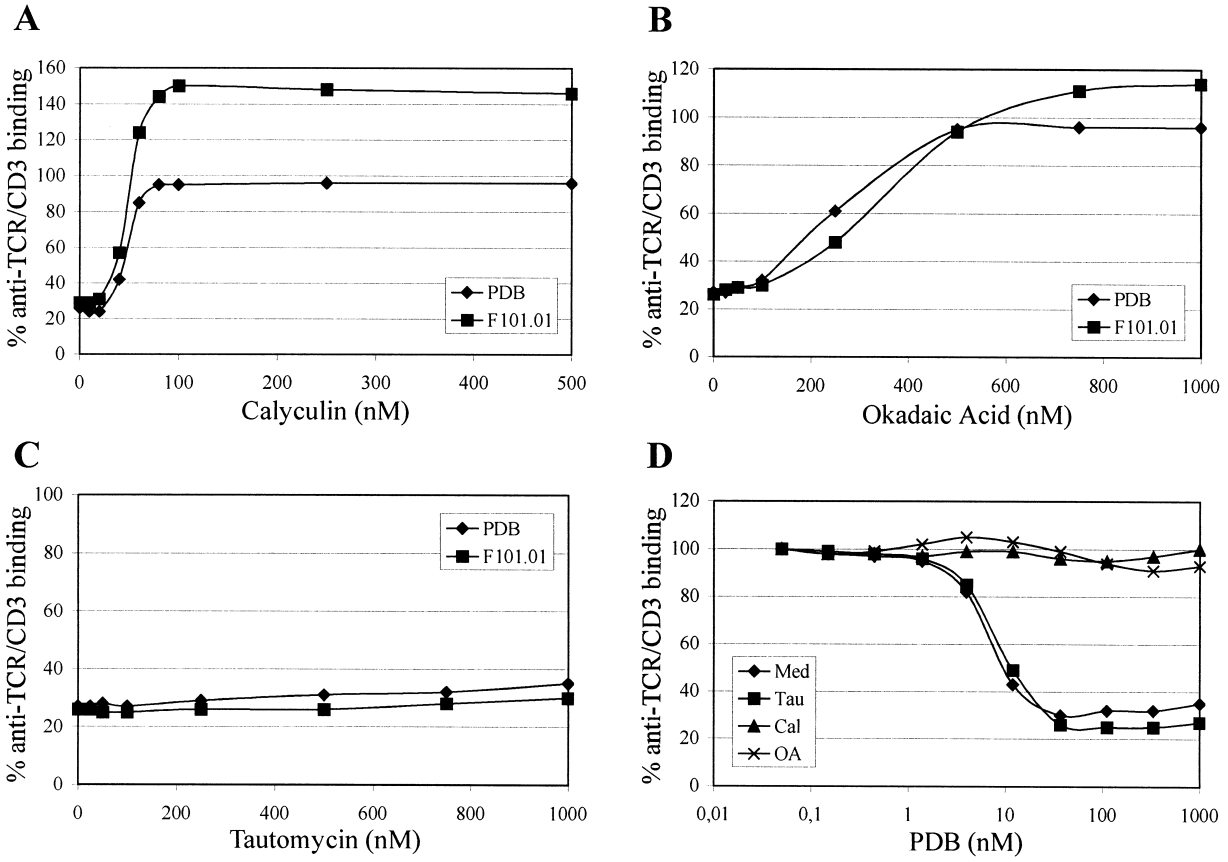

Fig. 5. Phosphorylation-induced T cell receptor endocytosis is blocked by PP2A inhibitors. Cells were incubated with calyculin A (A), okadaic acid (B), or tautomycin (C) at the indicated concentrations for 1 h at 37°C. The cells were subsequently incubated with PDB, the TCR ligand mAb F101.01, or with medium in the continuous presence of phosphatase inhibitors for one additional hour at 37°C. The cells were stained for FACS analysis as described in Section 2. Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent anti-TCR/CD3 binding (MFI of cells treated with PDB/F101.01 and phosphatase inhibitor)/(MFI of cells only treated with phosphatase inhibitor)×100%. (D) Cells were incubated with calyculin A (60 nM), okadaic acid (500 nM), tautomycin (1000 nM), or left untreated for 1 h at 37°C. The cells were subsequently incubated with various concentrations of PDB in the continuous presence of phosphatase inhibitors for one additional hour at 37°C. The cells were stained for FACS analysis as described above.

3.5. *Spontaneous endocytosis dependent on tyrosine- or leucine-based motifs is also blocked by PP2A inhibitors*

Although PP2A inhibitors blocked both ligand- and PKC-mediated TCR endocytosis we could not exclude the possibility that this effect was restricted to TCR phosphorylation. Therefore, we next looked at spontaneous endocytosis of receptors containing phosphorylation-independent receptor-sorting motifs. As examples we used the transferrin receptor and a chimeric CD4/3-SA molecule. Both receptors are endocytosed via clathrin-coated pits and vesicles [32,41]. The transferrin receptor is constitutively endocytosed dependent on a tyrosine-based (Tyr-Thr-Arg-Phe) endocytosis motif and the CD4/3-SA chimera is constitutively endocytosed dependent on a di-leucine-based (Asp-Lys-Gln-Thr-Leu-Leu) receptor-sorting motif [32]. Transferrin receptor endocytosis was measured by uptake of transferrin–fluorescein. The cells were pre-incubated with calyculin A (60 nM), okadaic acid (500 nM) or tautomycin (1000 nM) followed by incubation with transferrin–fluorescein for various periods. Transferrin uptake was subsequently measured by FACS analysis. Calyculin A and okadaic acid strongly inhibited transferrin uptake, whereas tautomycin did not show any inhibitory effect (Fig. 6A). Likewise, calyculin A se-

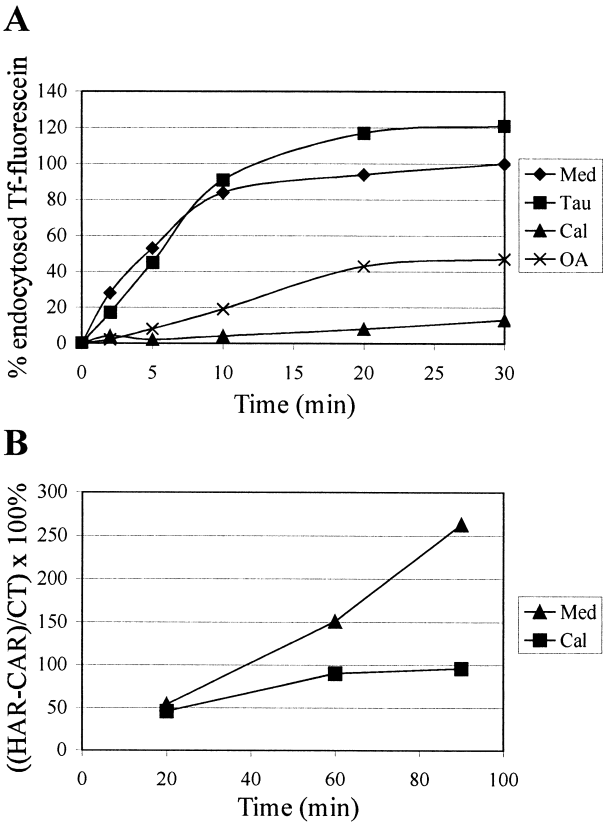

Fig. 6. Transferrin receptor and CD4/3-SA endocytosis is blocked by PP2A inhibitors. (A) Cells were incubated with calyculin A (60 nM), okadaic acid (500 nM), tautomycin (1000 nM), or left untreated for 1 h at 37°C followed by incubation with transferrin–fluorescein for various periods. MFI were recorded and used to calculate the percentage of endocytosed transferrin–fluorescein. (B) Cells were incubated with calyculin A (60 nM) or left untreated for 1 h at 37°C. The cells were prepared for FACS analysis as described in Section 2.

verely inhibited spontaneous CD4/3-SA endocytosis (Fig. 6B). Finally, fluid-phase endocytosis as measured by internalization of fluorescein–dextran was not reduced by the PP2A inhibitors (data not shown).

Taken together, these results demonstrated that PP2A inhibitors impaired clathrin-mediated endocytosis in general at concentrations which coincided with PP2A concentrations that inhibited β2-adaptin de-phosphorylation.

4. Discussion

In this study, we found that β2-adaptin is constitutively de-phosphorylated by PP2A and phosphorylated by a staurosporine-sensitive kinase in vivo. Furthermore, we found a correlation between β-adaptin phosphorylation and inhibition of clathrin-mediated endocytosis in general. Thus, treatment of cells with PP2A inhibitors at concentrations that inhibited β-adaptin de-phosphorylation abolished clathrin-mediated endocytosis. In contrast, treatment of cells with the PP1 inhibitor tautomycin did not inhibit de-phosphorylation of β-adaptin and did not affect clathrin-mediated endocytosis. Our results agree with previous studies which have indicated that PP2A plays a role in de-phosphorylation of coat proteins [30,31]. Thus, the mitotic inhibition of clathrin-coated vesicle invagination in vitro was reversed by diluting cytosol and this reversal was sensitive to okadaic acid [30]. Furthermore, de-phosphorylation of amphiphysin 1 and 2 in vitro was partially inhibited by okadaic acid [31]. We could not exclude that β-adaptin in untreated cells was phosphorylated to some extent. However, we could conclude that the mobility shift induced by PP2A inhibitors was caused by phosphorylation, as this shift was completely reversed by alkaline phosphatase treatment of the lysate. The mobility shift of β-adaptin was observed as shortly as 10 min after treatment of cells with PP2A inhibitors. Thus, cycles of de-phosphorylation/phosphorylation of β-adaptin must take place in vivo. Treatment of whole-cell lysate with PP2A inhibitor did not induce β-adaptin phosphorylation, demonstrating that the kinase responsible for β-adaptin phosphorylation was not active in the lysis buffer. This could be due to dilution of the cytosol as the lysis buffer did not contain ATP required for kinase activity.

In contrast to a previous study [25], we found that phosphorylated AP2 complexes were found at the plasma membrane using confocal laser scanning microscopy. This discrepancy might be explained by the use of different techniques and cells in determining AP2 distribution in these studies. Interestingly, we found that phosphorylated AP2 complexes were evenly distributed at the plasma membrane compared to non-phosphorylated AP2 complexes, which were predominantly found in aggregates. This could be explained by an impaired binding of phosphorylated AP2 with clathrin as previously described [25]. Thus, it could be speculated that although phosphor-

ylated AP2 complexes are recruited to the plasma membrane, they cannot bind to clathrin and consequently no aggregation of phosphorylated AP2 complexes is seen.

In addition to clathrin, phosphorylation of other components of the endocytosis apparatus like amphiphysin, Eps15, and epsin inhibits their binding to AP complexes [30,31,42]. From these studies, it should be expected that phosphorylation-induced inhibition of coat assembly would inhibit clathrin-mediated endocytosis in general. We extended these studies and found a close correlation between β-adaptin phosphorylation and impairment of clathrin-mediated endocytosis for three different receptors. Our results indicated that a common mechanism in clathrin-mediated endocytosis is impaired following treatment with PP2A inhibitors, as these receptors use different endocytosis motifs but are all endocytosed via clathrin-coated pits and vesicles. In agreement with previous studies [25,31,42], this could be induced by phosphorylation of β-adaptin and/or other proteins involved in clathrin-mediated endocytosis as the concentrations of PP2A inhibitors required to inhibit dephosphorylation of β-adaptin were identical to the concentrations required to inhibit endocytosis.

We found that a staurosporine-sensitive kinase is responsible for the constitutive phosphorylation of β-adaptin. Experiments using the PKA inhibitor KT5720 suggested that PKA, which has been implicated to take part in the endocytosis process, might be responsible for part of the phosphorylation [43,44]. PKA consensus phosphorylation sites are found in β-adaptin but they all reside outside the hinge domain, which is thought to bind clathrin. However, analyses using PKA specific activators and inhibitors seemed to exclude the possibility that PKA is involved in β-adaptin phosphorylation in vivo. Interestingly, KT5720 is a derivative of the broad-spectrum kinase inhibitor K252a that has been shown to inhibit phosphorylation of amphiphysin [31] and it is thus likely that the partial inhibition of β-adaptin phosphorylation by KT5720 is caused by cross-inhibition of other kinases than PKA. A kinase likely to participate in β-adaptin phosphorylation would be casein kinase II that has the ability to phosphorylate the clathrin b light chain in vitro [24,45]. However, we found that the casein kinase II inhibitor DRB did not inhibit β-adaptin phosphorylation indicating that casein kinase II is not involved in phosphorylation of β-adaptin in vivo. Thus, the exact kinase(s) involved in β-adaptin phosphorylation remains to be identified. The β-adaptin mobility shift assay presented in this study is a potent assay to study the effect of various kinase inhibitors and activators on β-adaptin phosphorylation and we are presently using this assay to further characterize the β-adaptin kinase.

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