Characterization of a protein phosphatase 2A holoenzyme (PP2A) that dephosphorylates the clathrin adaptors AP-1 and AP-2

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The AP-2 complex is a key factor in the formation of endocytic clathrin-coated vesicles (CCVs). AP-2 sorts and packages cargo membrane proteins into CCVs, it binds the coat protein clathrin and recruits numerous other factors to the site of vesicle formation. The structural information on the AP-2 complex and biochemical work has allowed to understand its function on the molecular level, and recent studies showed that cycles of phosphorylation are key steps in the regulation of AP-2 function. The complex is phosphorylated on both large subunits (α- and β2-adaptins) as well as at a single threonine residue (Thr-156) of the medium subunit μ2. Phosphorylation of μ2 is necessary for efficient cargo recruitment, while the functional context of the large subunit phosphorylation is unknown. Here, we show that the subunit phosphorylation of AP-2 exhibits striking differences, with half-lives of less than 1 min for μ2, ~ 25 min for β2 and ~ 70 min for α. We were also able to purify a phosphatase that dephosphorylates the μ2-subunit. The enzyme is a member of the protein phosphatase 2A family and composed of a catalytic Cβ subunit, a scaffolding Aβ subunit and a regulatory Bα subunit. RNAi knock-down of the latter subunit in Hela cells resulted in increased levels of phosphorylated adaptors and altered endocytosis, showing that a specific PP2A holoenzyme is an important regulatory enzyme in CCVs mediated transport.

INTRODUCTION

Internalization from the plasma membrane occurs by different pathways including uptake via clathrin-coated vesicles (CCVs). Although it is obvious that clathrin-coat assembly and disassembly need to be tightly regulated both spatially and temporally, little is known about the respective mechanisms that control the function of the many factors that are identified until now. However, increasing evidence suggests that cycles of phosphorylation/dephosphorylation are key events in the regulation of endocytic proteins (1). Indeed, the major endocytic CCV constituents, clathrin and AP-2 are functionally regulated by phosphorylation, as well as other factors including dynamin 1, amphiphysins, synaptojanin, AP180, epsin and eps15, which are collectively grouped as dephosphins, which become active in their dephosphorylated state (2).

Since AP-2 is a key factor in the formation of CCVs and known for long time to be phosphorylated, it is an ideal model-protein to analyze the functional consequences and regulatory mechanisms of cycles of phosphorylation (3). The complex is phosphorylated on the medium subunit μ2 at a single threonine residue (Thr-156), which is mediated in vitro by the two kinases AAK1 and GAK (4-8). Both kinases phosphorylate not only μ2, but also the μ1 subunit of the AP-1 complex in vitro, and it is yet unclear whether the two kinases act redundantly or distinctively (9,10). Beside this uncertainty, the current data shows that phosphorylation of the adaptor μ-subunits is a trigger for high-affinity binding to YxxΦ sorting signals of cargo membrane proteins (5,11).

While there is no evidence for phosphorylation of the AP-2 α2 subunit, both large subunits (α- and β2-adaptins) are phosphorylated, but it is not well established which residues are modified. Using a phospho-tyrosine specific antibody, the group of Alexander Sorkin identified an amino-terminal tyrosine (Tyr-6) as a major target site for B2 phosphorylation (12), while more recently others have identified Tyr-737 located in the ear domain as an agonist-dependent Src-kinase target (13). Not only tyrosine residues have been identified as phosphorylation target sites, but also serine/threonine kinases such as the recently

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identified CVAK104 (14) may impact on the phosphorylation status of β2-adaptin. Likewise, also α-adaptin is phosphorylated, but no data is available on the target sites and the involved kinase(s).

In order to get further insight into the role of AP-2 phosphorylation we used pulse/chase experiments to determine the phosphorylation turnover of the different AP-2 subunits. To our surprise the phosphorylation half-lives exhibited great differences, ranging from less than 1 min in case of μ2 to 25 min for β2 and around 70 min for the α subunit. In addition we purified a μ2-dephosphorylating enzyme activity from pig brain to homogeneity. The enzyme was identified as a protein phosphatase 2A (PP2A) and composed of all three subunits that constitute a functional holoenzyme: a catalytic Cß subunit, a scaffolding Aß subunit and a regulatory Bα subunit. Since the latter subunit is assumed to determine the intracellular location and substrate specificity, we knocked down the PP2A Bα subunit in HeLa cells by RNAi. In the affected cells, we detected increased levels of μ2 phosphorylation and altered endocytosis of transferrin, showing that a particular PP2A is an important regulatory enzyme in CCV mediated transport.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies** - Monoclonal anti-α-adaptin was from Alexis, antibodies against the PP2A subunits were from Santa Cruz (rabbit anti PP2A Cα/β; rabbit anti-PP2A Aα/β; rabbit anti-PP1; rabbit anti-PP2B) and Upstate (monoclonal anti-Bτ). Phosphorylated μ2 was detected with sheep antibodies (kindly provided by Elizabeth Smythe, University of Sheffield, UK), or with serum from a rabbit that was immunized with a synthetic peptide comprising phosphorylated Thr-156. Monoclonal antibodies against clathrin and γ-adaptin were from Transduction Labs, anti-Tubulin was from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies used for western-blotting and fluorochrome-labeled antibodies were from Dianova (Hamburg, Germany). Protein phosphatase 1, 2A and 2B proteins were obtained from Calbiochem. The serine/threonine kinase inhibitor staurosporine was from Sigma, the phosphatase inhibitors okadaic acid and calyculin-A were from Alexis.

**Cell culture** - HeLa and MDBK cells were grown at 37°C under standard cell culture conditions at 5% CO₂ in DMEM supplemented with 10% fetal calf serum. Cells derived from transfection with the PP2A B-α RNAi vector were maintained in the presence of 200 μg/ml G-418.

**Purification of CCVs and immunoisolation of AP-2** - The CCV preparation, the AP-2 purification from them, as well as the [³²P]-labeling of AP-2 and its immunoisolation were exactly as described (5,15).

**In vitro dephosphorylation of AP-2** - [³²P]-labeled AP-2 (10-15,000 cpm) was incubated in dephosphorylation buffer for 30 min at 37°C without any further additions (negative control) or with pig brain or HeLa cytosol (5 - 100 μg) or commercially protein phosphatase (0.05 U/sample) in a final volume of 100 μl. Subsequently, the samples were centrifuged, the supernatants were removed and the immunoadsorbed AP-2 was washed 3 times with dephosphorylation buffer followed by SDS-PAGE and subsequent detection with autoradiography and western-blotting.

**Purification of an AP-2-dephosphorylating enzyme from pig brain** - Pig brain cytosol obtained from the CCV purification was precipitated with (NH₄)₂SO₄ (35% final concentration), followed by centrifugation at 10,000 rpm for 20 min in a JA10 rotor (Beckman Coulter). The derived supernatant was dialyzed against buffer A (50 mM Tris pH 7.4; 2 mM MgCl₂; 0.1 mM EGTA; 1.5M (NH₄)₂SO₄), followed by centrifugation as described above. The fractionated cytosol was then applied to hydrophobic interaction chromatography on a Phenyl-Sepharose column (1x30 cm) equilibrated in buffer A and connected to a Perseptive Vision chromatography workstation at a flow rate of 4 ml/min. After sample application, the column was extensively washed for 1h with buffer A followed by gradient elution using buffer A without ammoniumsulfate. The μ2-phosphorylating activity was collected in the third of three peaks eluting at the end of the gradient. The fractions were pooled and dialyzed against buffer B (50 mM Tris pH 7.4, 2 mM MgCl₂, 0.1 mM EGTA, 5% Glycerol), followed by strong anion exchange chromatography using a 4.6x100 mm Source Q column at a flow rate of 5 ml/min. After washing of the column with 10 column volumes, proteins were eluted with a NaCl gradient (0 to 0.5 M within 15 column-volumes). The μ2-dephosphorylating activity eluted in two consecutive fractions at ~ 250 mM salt. The fractions were pooled, dialyzed against buffer B followed by chromatography on a 4.6x100mm DEAE-Sepharose column equilibrated in buffer B at a flow-rate of 1 ml/min. The μ2-dephosphorylating activity was collected in 2 fractions eluting at ~600 mM salt. Fractions were pooled (2 ml), concentrated to 200 μl by centrifugation and then applied to size exclusion chromatography using a 7.8x300 mm
G3000 SWXL column (Toso Haas) equilibrated in buffer B at a flow-rate of 0.5 ml/min. Only one major peak was detectable which contained the μ2-dephosphorylating activity and eluted at a size of ~200 kDa. The eluted protein sample was kept at 4°C until further use.

**Immunoprecipitation of cellular adaptors** – Pulse/chase experiments were used to immunoprecipitate intracellular phospho-AP-2. In brief, confluent cells on 3 cm dishes were starved in phosphate-free medium followed by incubation with 220 μCi [32P]orthophosphate in 1 ml phosphate-free DMEM (5% serum) for 8.5 h (pulse). Afterwards, the labeling medium was removed, the cells were washed and subsequently incubated for the indicated chase periods in DMEM containing 10-fold excess phosphate. Control cells (t = 0 min) were collected after the pulse. All incubations were stopped by transferring the cells on ice and immediately exchanging the media for ice-cold homogenisation buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, pH=7.2). The cells were scraped in 1 ml of homogenisation buffer, collected by centrifugation for 5 min with 500 × g, then resuspended in buffer B (0.5 M Tris, 150 mM NaCl, 1 mM EDTA, pH=7.2, 1% Triton-X-100, 10 mM calyculin, 10 mM okadaic acid, 400 mM cantharidin, 900 mM endothal protease inhibitors), incubated for 30 min followed by centrifugation for 30 min at 100,000 × g to remove insoluble proteins. AP-2 was immunoprecipitated from the supernatants as described (5) and resolved by conventional or 6 M urea containing SDS-PAGE (16). The subunit incorporated radioactivity was quantified from the dried gels using a FUJIX BAS 1000. In some experiments AP-1 was precipitated as described above, using monoclonal anti γ-adaptin antibodies.

The amount of radioactivity present in the labeling media before/after the pulse and in the media after the chase was determined by Cerenkov-Counting. By subtracting the values, the intracellular radioactivity after the chase was determined. For each experiment, the sums over time of the values of α-, β2-, μ2-phosphorylation and intracellular radioactivity were determined. To level these values from different experiments, they were multiplied with a factor, so that the corresponding sums in each experiment were the same. Average values and standard deviations were then generated and expressed in percent of the control average values (t = 0 min). Intracellular radioactivity was interpolated with the sum of two e-functions, differentiated and the differentiation was expressed in percent of control, too. The phosphorylation half-lives were determined as described in the supplementary information. For the data evaluation Microsoft Excel and R program (www.r-project.org) were used. Data was interpolated with the formulas after the method of least squares using the function "nls" in the R program.

**Identification of proteins by mass spectrometry** - For peptide mass fingerprint analysis, the respective protein bands visible after Coomassie staining were excised, cut in 1 mm small pieces and subjected to in-gel digestion with trypsin (17), desalted on C18 ZipTip, and analyzed by MALDI-TOF mass spectrometry using dihydrobenzoic acid as matrix and two autolytic peptides of trypsin (m/z 842.51 and 2211.10) as internal standards. The mass spectrometric data were used by Mascot search algorithm for protein identification in the NCBI nr protein database.

**Suppression of PP2A Bα by RNA interference** - A plasmid-based system was used to stably suppress the expression of the PP2A Bα subunit in HeLa cells. The sequence GAACCGACTTTCTGCTTAG starting at position 1667 in the 3′ untranslated region of the human Bα mRNA (NM_002717) was used as a target. In brief, two complementary oligonucleotides were synthesized containing the above target sequence followed by a spacer of 9 nucleotides, the target sequence in reverse orientation and a sequence of 5 thymidine bases as a terminator of transcription. Both primers were annealed and cloned into the pSHH plasmid (BIOCARTA, Carlsbad, USA) using SalI and XbaI restriction sites. The plasmid that also confers resistance to G418 was transfected into HeLa cells using Effectene (Qiagen) as a transfection reagent. Single clones were assayed by western-blotting for suppression of the Bα subunit. Three clones in which Bα expression was reduced by more than 80% were used for further analysis. No obvious variation between the clones was noticeable. To rule out unwanted side effects of vector-based RNAi for the PP2A Bα subunit, we also used siRNA oligos for the same and an additional target sequence (GTCTCATAGCAGAGGAGAA).

For rescue experiments, the coding sequence of the human Bα-cDNA was cloned in frame into the pCMV-3Tag-6 vector (Stratagene) using HindIII and XhoI restriction sites. The final insert codes for an amino-terminal triple repeat of the FLAG tag (25 amino acids) followed by an additional spacer sequence (30 amino acids) that was found to be essential for detection of the tag-

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sequence. Expression from the vector in HeLa cells was not sensitive to the target sequence of vector-based RNAi and the respective siRNA oligos, because the target sequences in the 3’-untranslated region were deleted from the insert. The recombinant 3xFLAG-tagged βc subunit was detected by using tag-specific monoclonal (GenScript) or polyclonal (Delta Biolabs) antibodies. Integration of the tagged recombinant βc subunit into PP2A holoenzymes was verified by detection of the PP2A C- and A-subunits in anti-FLAG antibody immunoprecipitates and by detection of the recombinant subunit after pulldown of PP2A using agarose-immobilized microcystin (see supplementary figure 1).

Endocytosis experiments – Cells on glass coverslips were serum-starved for 1h in DMEM with 0.5% serum albumin, washed with PBS and incubated for 15 min on ice in the presence of 20 µg/ml Alexa 546-conjugated human transferrin (Tf), human Dil-LDL (20 µg/ml) or Rhodamine-labeled dextran (2 mg/ml). Afterwards, the cells were either immediately fixed and processed for microscopy or first incubated at 37°C for 5 min up to 2 hours to monitor internalization.

For the biochemical analyses of Tf uptake, we used the biotinylated protein. Parallel samples were incubated on ice with Biotin-Tf for 30 min followed by washing with PBS. Then one sample was collected for subsequent analysis of the total amount of bound Tf. A second sample was left on ice and later subjected to Trypsin treatment to control the removal of surface Tf. All other samples were incubated at 37°C for up to 10 min, then chilled on ice, washed with PBS and incubated 3 times for 5 min with Trypsin to remove surface Tf. After washing with PBS, 1% serum, the cells of all samples were scraped, collected by centrifugation and homogenised in PBS, 1% Triton X-100. Subsequently, the Biotin-Tf was immunoprecipitated with Streptavidin-agarose and detected by western-blotting. A concentration series of biotin-Tf served as a control for the amount of cell-internalised protein. Trypsin treatment was able to remove more than 95% of all surface Tf. Endocytosis is expressed as the amount of cell-associated Biotin-Tf relative to the total amount initially bound on ice.

Immunofluorescence - Cells were seeded on glass coverslips one or two days before use. After fixation with 3% paraformaldehyde for 20min, blockage of remaining aldehyde groups with 50mM NH₄Cl in PBS for 10min, the cells were permeabilized using 0.5% saponin in PBS. Incubation with the primary antibodies was carried out for 1h at 37°C, followed by incubation for 15min in 10% goat serum and incubation with the fluorochrome-conjugated secondary antibodies for 30min at 37°C. After washing with PBS and water, the samples were mounted in Fluoromount (DAKO) and analyzed with a confocal laser scanning microscope (Leica TCS-2 AOBS). For quantitative analysis we determined the fluorescence intensity along identical longitudes of plasma membrane profiles in an area of 200 µm x 200 µm in HeLa and RNAi cells.

Miscellaneous - For inhibition of µ2-dephosphorylation by PP2A, a synthetic Thr-phosphorylated peptide was used (-CQ148SQITSQVTGQIGWR162) that resembles the phosphorylation site in the AP-2 µ2 subunit with an additional cysteine at the amino-terminus. The equivalent non-phosphorylated peptide and two other non-related peptides were used as controls.

RESULTS AND DISCUSSION

The AP-2 subunits exhibit different phosphorylation turn-over rates - In order to gain insight into the phosphorylation turn-over of the different AP-2 subunits, cells were labeled with [32P]-phosphate for 8.5 hours to mark a large pool of proteins. After labeling, AP-2 was either immediately immunoprecipitated (t = 0 min), or the cells were chased for different periods of time in phosphate-rich medium before AP-2 was collected. Subsequent to immunoprecipitation, AP-2 was subjected to SDS- and urea-containing SDS-PAGE to resolve α-, β2- and µ2-adaptins (Fig. 1A). Finally, the incorporated [32P]-phosphate was quantified after autoradiography and the amount of subunit-incorporated phosphate relative to the beginning of the chase (t = 0 min) was plotted together with the export velocity of [32P]-inorganic phosphate from the cells (red curve in Fig. 1B). Interestingly, we noticed a faster phosphorylation turn-over of µ2 compared to β2 and α-adaptin. In order to calculate realistic phosphorylation turn-over rates from the raw data shown in figure 1A, the final mathematical equations had to consider the continuously decreasing probability of [32P]-protein re-phosphorylation during the chase period (see supplementary information for a detailed description). For the turn-over of µ2 phosphorylation we calculated a half-life of less than 1 min, while the two large adaptor subunits exhibited half-lives of ~25 min (β2-adaptin) and ~70 min (α-adaptin). The calculated phosphorylation half-life of µ2 fits well to the turn-over rate of a forming clathrin-coated vesicle (18,19), which includes a cycle of µ2.
phosphorylation and dephosphorylation in the process of high affinity binding to tyrosine-based sorting signal containing membrane cargo proteins (11).

The phosphorylation turn-over of the two AP-2 large subunits was much slower as compared to the μ2 subunit. In this context it is important to note that we did not discriminate between the potential amino acid residues involved. Using phosphoamino acid analysis, Wilde and Brodsky demonstrated phosphorylation of α- and β2-adaptin only on serine residues, while phosphorylation of tyrosine or threonine residues was not detectable (20). However, the pattern of phosphorylation may change upon the status of the cells analyzed. Recent data showed that stimulation of cells with EGF resulted in phosphorylation of β2-adaptin at Thr-156 as a substrate (see Material and Methods for details). Incubation of the proteins was identified as actin (asterisk in Fig. 2A), the other three proteins were identified as the Aß, Bα and Cß subunits of PP2A. In conclusion we succeeded in the first purification of a PP2A holoenzyme that mediates dephosphorylation of the μ2 subunit of AP-2. Our data support the hypothesis of a phosphatase dependent on divalent metal ions or a tyrosine-phosphatase (not shown). In contrast, calyculin-A, microcystin and oacitic acid totally abolished μ2 dephosphorylation at nanomolar concentrations (not shown), indicating that a trimeric protein phosphatase like PP1 or PP2A may be a more likely candidate. Indeed, PP2A was already characterized as the enzymatic activity that mediates dephosphorylation of the μ1 subunit of the AP-1 complex (23), but no specific enzyme was identified. Because most phosphatases are predominantly cytosolic we used pig brain cytosol as a starting material for the purification of the μ2-dephosphorylating enzyme. Amoniumsulfate precipitation was first used to deplete the cytosol of a substantial number of proteins. Under these conditions, the enzyme remained in the soluble fraction as verified by dephosphorylation of AP-2 using the assay described above. The fractionated cytosol was then applied to hydrophobic interaction chromatography, followed by collection of those fractions that contained μ2-dephosphorylating enzymatic activity. The pooled fractions were further applied to strong and weak anion exchange chromatography and finally to size exclusion chromatography. At this step the μ2-dephosphorylating activity eluted in a single small peak that contained four proteins (Fig. 2B), which were subjected to peptide mass fingerprinting. With mouse scores ≥ 200, one of the proteins was identified as actin (asterisk in Fig. 2C), the other three proteins were identified as the Aß, Bα and Cß subunits of PP2A. In conclusion we succeeded in the first purification of a PP2A holoenzyme that mediates dephosphorylation of the μ2 subunit of AP-2. The purified enzyme preparation was free of any contamination by PP1 or PP2B as verified by western-blotting, while each PP2A subunit was unequivocally detected by antibodies of the respective specificity (Fig. 2B). Based on the protein-specific activity towards μ2, our purification resulted in a more than 5,000-fold enrichment of the PP2A holoenzyme. Between different preparations we noticed that the PP2A A- and C-subunits were mostly present in AP-2 prior and after cytosol incubation were used for the detection of α- and μ2-adaptin and revealed that equal amounts were present, clearly demonstrating that the loss of signal in the autoradiogram was the result of dephosphorylation, ruling out loss of AP-2 in the assay. When the cytosol was preincubated with sodium-orthovanadate or EDTA/EGTA, dephosphorylation of μ2 was still effective, arguing against the involvement of a phosphatase dependent on divalent metal ions or a tyrosine-phosphatase (not shown). 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variants, the theoretical number of PP2A holoenzymes exceeds 70, each consisting of one of the two A-subunits (α or β), one of the two catalytic C-subunits (α or β) and one of the many B-subunits (26-28). Because the holoenzyme purified by us contained the Bα subunit of the B55 family (29), it was the obvious knockdown target in HeLa cells. As shown in figure 4A, we could efficiently and specifically suppress Bα expression (~5% residual protein), while neither the PP2A catalytic subunit nor other control proteins were affected. As a control for the specificity of suppression of the Bα subunit, we generated a recombinant subunit that was insensitive to RNAi, because the cDNA was lacking the RNAi target sequences located in the 3' untranslated region. The recombinant tagged Bα subunit had a 50 amino acid aminoterminal extension including a 3xFLAG tag and was expressed in Bα RNAi cells (rescue) as confirmed by detection with FLAG-tag specific antibodies (Fig. 4A, lane 3). As shown with antibodies against endogenous Bα, the recombinant subunit has a slightly higher molecular mass, was exclusively detectable in the rescue cells and was slightly overexpressed (compare lanes 1-3 in Fig. 4A). We next labeled cells with [32P]-phosphate and used equal amounts of protein for precipitation of AP-2. As indicated in Fig. 4B, the phospho-μ2 signal was increased by 2-fold in AP-2 derived from the RNAi cells, even though the same amounts of AP-2 were gained from all cell types. Note that the original degree of μ2 phosphorylation was restored in RNAi cells expressing the recombinant 3xFLAG Bα subunit, indicative of its integration into functional PP2A holoenzymes which dephosphorylate AP-2. This notion was further corroborated by co-immunoprecipitation of the PP2A A- and C-subunits with FLAG-tag specific antibodies and by detection of the recombinant Bα subunit in a pulldown with agarose-immobilized microcystin, which was used as an affinity matrix for the capture of PP2A (see supplementary figure 1). We therefore conclude that the pool of AP-2 phosphorylated at μ2 is enlarged in Bα RNAi cells. Consistent with this result, the cytosol from RNAi cells lost its μ2-dephosphorylating activity (Fig. 4C) as compared to cytosol from HeLa cells and rescue cells expressing recombinant Bα, or after incubation with the purified PP2A. In the same set of experiments we used [32P]-labeled AP-1 as a substrate, which was also dephosphorylated by the purified PP2A, but not by the RNAi cell derived cytosol. This observation is consistent with the results of the Kornfeld group, who previously showed that dephosphorylation of the

**Knockdown of PP2A Bα affects the phosphorylation status of μ2** - The results described above show that the purified PP2A holoenzyme mediates μ2 dephosphorylation in vitro. To analyze the significance of our findings for living cells, we suppressed the expression of the PP2A Bα subunit in HeLa cell by vector-based RNAi. In this context one should note that the highly conserved PP2A A-subunit associates with the C-subunit in a dimeric core enzyme that is thought to lack specificity for substrate and intracellular localization (24,25). Both functions are mediated by the B-subunit family of proteins. Four such families have been characterized, each comprising four or more members. Since some of the B-proteins can be expressed as splice variants, the theoretical number of PP2A holoenzymes exceeds 70, each consisting of one of the two A-subunits (α or β), one of the two catalytic C-subunits (α or β) and one of the many B-subunits (26-28). Because the holoenzyme purified by us contained the Bα subunit of the B55 family (29), it was the obvious knockdown target in HeLa cells. As shown in figure 4A, we could efficiently and specifically suppress Bα expression (~5% residual protein), while neither the PP2A catalytic subunit nor other control proteins were affected. As a control for the specificity of suppression of the Bα subunit, we generated a recombinant subunit that was insensitive to RNAi, because the cDNA was lacking the RNAi target sequences located in the 3'-untranslated region. The recombinant tagged Bα subunit had a 50 amino acid aminoterminal extension including a 3xFLAG tag and was expressed in Bα RNAi cells (rescue) as confirmed by detection with FLAG-tag specific antibodies (Fig. 4A, lane 3). As shown with antibodies against endogenous Bα, the recombinant subunit has a slightly higher molecular mass, was exclusively detectable in the rescue cells and was slightly overexpressed (compare lanes 1-3 in Fig. 4A). We next labeled cells with [32P]-phosphate and used equal amounts of protein for precipitation of AP-2. As indicated in Fig. 4B, the phospho-μ2 signal was increased by 2-fold in AP-2 derived from the RNAi cells, even though the same amounts of AP-2 were gained from all cell types. Note that the original degree of μ2 phosphorylation was restored in RNAi cells expressing the recombinant 3xFLAG Bα subunit, indicative of its integration into functional PP2A holoenzymes which dephosphorylate AP-2. This notion was further corroborated by co-immunoprecipitation of the PP2A A- and C-subunits with FLAG-tag specific antibodies and by detection of the recombinant Bα subunit in a pulldown with agarose-immobilized microcystin, which was used as an affinity matrix for the capture of PP2A (see supplementary figure 1). We therefore conclude that the pool of AP-2 phosphorylated at μ2 is enlarged in Bα RNAi cells. Consistent with this result, the cytosol from RNAi cells lost its μ2-dephosphorylating activity (Fig. 4C) as compared to cytosol from HeLa cells and rescue cells expressing recombinant Bα, or after incubation with the purified PP2A. In the same set of experiments we used [32P]-labeled AP-1 as a substrate, which was also dephosphorylated by the purified PP2A, but not by the RNAi cell derived cytosol. This observation is consistent with the results of the Kornfeld group, who previously showed that dephosphorylation of the
Membrane protein binding in the process of clathrin-coated vesicle formation at the plasma membrane is one of the important functions of AP-2. We therefore analyzed the localization of clathrin and AP-2 in HeLa and PP2A α-subunit RNAi cells and monitored whether RNAi had any impact on endocytic processes. As shown in figure 5, we noticed that the typical punctuate pattern of AP-2 and clathrin was more pronounced in the RNAi cells (compare C to F). Interestingly, the surface expression of the Tf receptor, which is widely used as a readout for functional analyses of endocytic proteins (30-32) was also increased by 2-fold in the RNAi cells as indicated after incubation with fluorescent transferrin on ice (compare Fig. 5 A and B, quantification in G). The endocytosis of transferrin reached similar levels (60 % within 6 min) in HeLa and RNAi cells, but the latter exhibited a significant higher rate of uptake (Fig. 5D). The amount of intracellular detectable transferrin slightly decreased between 6 min and 8 min, most likely because some transferrin already recycled back to the cell surface within this period. In contrast to our findings on the internalization of transferrin, the receptor-mediated uptake of fluorescent LDL and the fluid-phase endocytosis of Rhodamine-labeled dextran were not significantly affected when analyzed by immunofluorescence (not shown). In conclusion, our results show that knockdown of the PP2A α-subunit affects the amount of AP-2 and clathrin that was localized to coated structures, however endocytic processes were only marginally affected. One explanation might be that imbalances in the regulatory cycle of AP-2 phosphorylation and dephosphorylation may only have a small impact on the endocytic machinery in general. Still we are just beginning to explore the mechanisms, by which kinases and phosphatases regulate the functions of endocytic proteins. Several kinases as well as PP1, PP2A and other phosphatases are present in coated vesicle preparations (33-36), suggesting that the activities of these counteracting enzymes have to be tightly controlled within the short period of coated vesicle formation. Interestingly, the kinase AAK1 is active in a protein preparation liberated from clathrin-coated vesicles by Tris extraction and easily phosphorylates AP-2 when ATP is added. In contrast, the PP2A in the same preparation is inactive and unable to dephosphorylate AP-2 (Ricotta and Höning, unpublished). Whether the PP2A activity is regulated by posttranslational modification(s), how the enzyme is recruited to forming clathrin-coated vesicles and whether it has other substrates apart from AP-2 are some of the questions that we need to address to get a deeper insight into the functional regulation of endocytosis.

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References

FIGURE LEGENDS

FIGURE 1. Phosphorylation turnover of AP-2 subunits. AP-2 was immunoprecipitated from cells that had been pulse labeled with [32P]-phosphate, or after pulse labeling and chase periods of the indicated duration. A: Autoradiogram derived from SDS and urea-SDS PAGE showing the time-dependent decay of AP-2 subunit phosphorylation. B: Quantification of the data shown in A. The subunit-incorporated [32P]-phosphate after labeling (0 min) was set to 100%, all other values were related to that. The error bars indicate the variation between 4 independent experiments, the black traces represent model simulations of half-lives ranging from 5 to 60 min.

FIGURE 2. Identification of a µ2-dephosphorylating phosphatase. A: AP-2 phosphorylated on µ2 was obtained by immunosolubulation from Tris-extracted CCVs, which had been incubated with [32P]-γATP. Incubation with pig brain cytosol resulted in substantial dephosphorylation of µ2. Westernblot analysis for α- and µ2-adaptin revealed that no AP-2 was lost during the incubation. B: The µ2-dephosphorylating enzymatic activity was purified by chromatography from pig brain cytosol. All fractions of each step were tested for µ2-dephosphorylation using the assay described in A. Positive fractions were pooled and subjected to the next purification step. The final size exclusion chromatography yielded in a pool of activity, which was recovered from 2 fractions eluting with an molecular mass ~ 200 kDa. C: The Coomassie stain of the purified phosphatase activity is shown in the first lane. Three of the purified proteins were identified by peptide mass fingerprinting as the Aß, Bα and Cß subunits of a PP2A holoenzyme, protein 4 (asterisk) was identified as actin. Westernblot analysis of the purified enzyme revealed the absence of PP1 (lane 2) and PP2B/calcineurin (lane 3), while the 3 subunits of PP2A were positively identified (lane 4, the specificity of the antibodies is indicated on the right).

FIGURE 3. Characterisation of the µ2-dephosphorylating PP2A. The purified PP2A holoenzyme was compared with commercially available dimeric and trimeric forms of PP2A in the µ2 dephosphorylation assay. Note that only the trimeric holoenzyme was active (A). To further proof that dephosphorylation of µ2 was mediated by the purified PP2A and not by a contaminating enzyme, a 1µg sample of the PP2A preparation (input) was subjected to depletion with either control IgG beads or beads coated with a monoclonal antibody specific for the PP2A catalytic subunit (B). After depletion, the beads were collected and tested for PP2A by western-blotting using 2 different antibodies. No PP2A was captured by control beads, but only by anti PP2A-beads. The supernatants of the depletion and a non-treated control were used for µ2 dephosphorylation. Only the supernatants of the control and the IgG beads were active, while that of the anti-PP2A beads lost all µ2-phosphorylating activity. As shown in C and D, the activity of the purified enzyme was inhibited by increasing concentrations of two known PP2A inhibitors (C) and by addition of a synthetic Thr-156 phosphorylated µ2-peptide, but not by a non-related peptide (D), demonstrating that the AP-2 µ2-subunit is a substrate of the purified PP2A holoenzyme.

FIGURE 4. Knockdown of PP2A Bα impairs µ-subunit dephosphorylation. As shown in A, expression of the endogenous Bα subunit of PP2A was efficiently knocked down in HeLa cells by RNAi (see Material and Methods for details), while three other marker proteins were not affected. Rescue cells express a recombinant, RNAi insensitive 3xFLAG tagged Bα subunit as indicated by the slight shift in mobility and its detection by anti-FLAG antibodies (compare lanes 2 and 3). When equal amounts of AP-2 (westernblot in B) were precipitated from [32P]-labeled cells, we noticed a 2-fold increase of phospho-µ2 in RNAi cells (radiography in B), suggesting an inhibition of the involved PP2A. Expression of the RNAi-insensitive 3xFLAG tagged Bα was sufficient to restore the original phosphorylation pattern of µ2. In support of these results, cytosol prepared from Bα RNAi cells was impaired in µ2-dephosphorylation compared to cytosol from rescue cells and HeLa cells or to incubation with the purified PP2A (C). Note that PP2A does not only dephosphorylate µ2 but also the...
AP-1 μ1 subunit. The numbers represent the remaining amount phospho-μ substrate after cytosol incubation as % of the untreated control in lane 1.

**FIGURE 5. Knockdown of PP2A Bα perturbs transferrin endocytosis.** HeLa cells (A, C, E) and PP2A Bα RNAi cells (B, D, F) were incubated on ice with Alexa546-conjugated human transferrin (A and B) to label the transferrin receptor population present at the plasma membrane. Note that the cell surface staining intensity was stronger in the RNAi cells. Additionally, the punctuate pattern of the two main CCV proteins clathrin (C and D) and AP-2 (E and F) was more pronounced, indicative of a higher number of coated structures. Quantification of the transferrin signal intensities along plasma membrane profiles revealed a more than 2-fold increase in the amount of at the plasma membrane (G). Consistent with this finding, the uptake of biotinylated transferrin in RNAi cells was faster in the initial phase of endocytosis (H). A fraction of the cells was analyzed by western-blotting to verify the effectiveness of RNAi (I).
FIGURE 1
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5