Phosphorylation of the medium chain subunit of the AP-2 adaptor complex does not influence its interaction with the tyrosine based internalisation motif of TGN38

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Abstract Tyrosine based motifs conforming to the consensus YXXΦ (where Φ represents a bulky hydrophobic residue) have been shown to interact with the medium chain subunit of clathrin adaptor complexes. These medium chains are targets for phosphorylation by a kinase activity associated with clathrin coated vesicles. We have used the clathrin coated vesicle associated kinase activity to specifically phosphorylate a soluble recombinant fusion protein of μ2, the medium chain subunit of the plasma membrane associated adaptor protein complex AP-2. We have tested whether this phosphorylation has any effect on the interaction of μ2 with the tyrosine based motif containing protein, TGN38, that has previously been shown to interact with μ2. Phosphorylation of μ2 was shown to have no significant effect on the in vitro interaction of μ2 with the cytosolic domain of TGN38, indicating that reversible phosphorylation of μ2 does not play a role in regulating its direct interaction with tyrosine based internalisation motifs. In addition, although a casein kinase II-like activity has been shown to be associated with clathrin coated vesicles, we show that μ2 is not phosphorylated by casein kinase II implying that another kinase activity is present in clathrin coated vesicles. Furthermore the kinase activity associated with clathrin coated vesicles was shown to be capable of phosphorylating dynamin 1. Phosphorylation of dynamin 1 has previously been shown to regulate its interaction with other proteins involved in clathrin mediated endocytosis.

Key words: Clathrin; Adaptor; Phosphorylation; Tyrosine motif; Dynamin

1. Introduction

A large proportion of membrane traffic events in eukaryotic cells involve the formation of coated vesicles at the donor membranes (reviewed in [1]). Many different coat complexes involved in vesicle formation have been isolated in recent years but the clathrin coat remains the most well characterised complex to date (reviewed in [2]). Clathrin coated vesicles form at the plasma membrane and facilitate such processes as receptor mediated endocytosis and nutrient uptake. Clathrin coats also form at the *trans*-Golgi network (TGN) where they play a role in sorting lysosomal enzymes to the endocytic pathway and secretory granule biogenesis. The formation of clathrin coats involves the organisation of individual clathrin molecules, triskelion in shape, into cage-like lattices. This polymerisation of clathrin molecules is catalysed by heterotetrameric adaptor protein (AP) complexes that link the cytosolic domains of integral membrane proteins to the clathrin coat [2,3]. The AP complexes consist of two large subunits (of approximately 100 kDa), one medium chain (μ) subunit, and one small (σ) subunit. The most well characterised AP complexes are AP-1, associated with vesicle budding at the TGN, and AP-2, associated with vesicle formation at the plasma membrane. The interaction of the AP complexes with clathrin appears to occur via the hinge region of the β chain adaptin subunits (β1 and β2 in AP-1 and AP-2), interacting with the most distal region of the clathrin leg (terminal domain) [4]. The μ chain subunits of the AP complexes have been shown to interact directly with the cytosolic domains of some integral membrane proteins thus providing the mechanism by which adaptors link the clathrin coat to those integral membrane proteins [5]. The μ chains interact with tyrosine based motifs (present within the cytosolic domains of these integral membrane proteins) that conform to the consensus YXXΦ (where Φ represents a bulky hydrophobic residue), this motif being known to mediate such events as endocytosis, lysosomal targeting and basolateral targeting in polarised cells [6]. The interactions of the different μ chains with various integral membrane proteins and their tyrosine based motifs have been extensively characterised in recent years [7–13].

Various subunits of the adaptor complexes and clathrin have been shown to be phosphorylated in vivo; it has also been shown that these phosphorylation events may have a role in regulating clathrin assembly [14]. These data showed that the two most extensively phosphorylated subunits are the β1 and β2 adaptin subunits and that they are phosphorylated on serine residues in their hinge region. Both in vitro and in vivo analysis suggested that this phosphorylation prevents reassociation of the adaptor complexes with clathrin and thus may have a role in the control of clathrin coat disassembly and reassembly. In addition to the β chain subunits, the μ chain subunits were also found to be targets of phosphorylation [14]. No data were presented on the role of μ chain phosphorylation, but it has subsequently been commented that it would be of interest to assess the effect of phosphorylation on interaction of adaptors with integral membrane protein cytosolic domains [15]. The published data suggest that the kinase(s) involved in the phosphorylation of the adaptor subunits is/are physically associated with the clathrin coated vesicles themselves. In addition, recent reports have also demonstrated a role for phosphorylation of other components involved in the formation of clathrin coated vesicles in endocytosis. Proteins including dynamin 1, synaptojanin

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Abbreviations: TGN, *trans*-Golgi network; AP, adaptor protein; CKII, casein kinase II; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; IPTG, isopropyl-β-D-thiogalactopyranoside; TRX, thioredoxin

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and amphiphysin have been shown to be phosphorylated (by unknown kinase(s) in vivo) and data suggest that these phosphorylation events prevent interaction of these proteins with each other and other components of the clathrin coat [16].

The cytosolic domain of the integral membrane protein TGN38 contains a tyrosine based motif, SDYQRL, which has been shown to interact with high affinity with the medium chain subunit of the AP-2 adaptor complex (μ2) [7,11]. We now show that proteins stripped from purified clathrin coated vesicles contain a kinase activity that specifically phosphorylates a recombinant soluble fusion protein of μ2. The proteins stripped from the clathrin coated vesicles contain a casein kinase II (CKII)-like activity but the μ2 kinase is unlikely to be CKII itself because recombinant CKII does not phosphorylate μ2. In addition, we have tested the effect of the phosphorylation of μ2 on the interaction between μ2 and the cytosolic domain of TGN38. Our data show that the phosphorylation of μ2 has no significant effect on interaction with TGN38 in vitro, and thus indicate that the reversible phosphorylation of μ2 is unlikely to play a direct role in the in vivo regulation of the interaction between AP-2 and tyrosine based internalisation motifs. Furthermore we have shown that the clathrin coated vesicle associated kinase(s) activity can also phosphorylate dynamin 1 and synaptojanin.

2. Materials and methods

All reagents were purchased from Sigma (Poole, UK) unless otherwise stated.

2.1. Preparation of clathrin coated vesicles

Clathrin coated vesicles were purified from rat brains essentially using the method described in [17]. Briefly, rat brains were homogenised in isolation buffer (100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol) with the addition of protease inhibitors (1 μg ml^{-1} leupeptin, 2 μg ml^{-1} antipain, 10 μg ml^{-1} benzamidine, 10 units ml^{-1} aprotinin, 1 μg ml^{-1} chymostatin, 1 μg ml^{-1} pepstatin A, 1 mM PMSF). The homogenate was centrifuged (10 000 rpm, 10 min, 4°C, SS-34 rotor (Sorvall)). The supernatant was centrifuged (34 000 rpm, 60 min, 4°C, TFT-65.13 rotor (Sorvall)) and the resulting pellet resuspended in 10 ml isolation buffer plus protease inhibitors and an equal volume of Ficoll/sucrose solution added (12.5% Ficoll 400, 12.5% sucrose in isolation buffer). The suspension was centrifuged (19 000 rpm, 40 min, 4°C, SS-34 rotor), the supernatant diluted 10-fold with isolation buffer and centrifuged (34 000 rpm, 30 min, 4°C, TFT-65.13 rotor). The pelleted clathrin coated vesicles were resuspended in isolation buffer, snap frozen in liquid nitrogen and stored at −80°C.

2.2. Stimulation of clathrin coated vesicle associated kinase activity

Proteins were stripped from the clathrin coated vesicles by incubating the purified vesicles in an equal volume of 1 M Tris, pH 7.0, 30 min, 0°C, and stripped membranes removed by centrifugation (34 000 rpm, 30 min, 4°C, TFT-65.13 rotor). Kinase activity was stimulated as essentially described in [18]. Briefly, stripped proteins were diluted 1 in 5 into clathrin coated vesicle-associated kinase buffer (final concentrations: 150 mM KCl, 5 mM $MgCl_2$, 100 μg ml^{-1} polylysine, 100 μM ATP, ±9.25 MBq ml^{-1} [γ-^{32}P]ATP) and incubated with target substrates for 1–2 h at room temperature. Reactions were stopped by addition of EDTA to a final concentration of 20 mM or by boiling in SDS-PAGE sample buffer (200 mM Tris-HCl, pH 6.8, 25% v/v glycerol, 5% w/v SDS, 0.1% w/v bromophenol blue).

2.3. Casein kinase II phosphorylation

Proteins were incubated with 0.05 mU of casein kinase II (Boehringer Mannheim, Lewes, UK) in CKII buffer (20 mM MES, pH 6.9, 130 mM KCl, 10 mM $MgCl_2$, 4.8 mM dithiothreitol) in a 10 μl reaction in the presence of 0.37 MBq [γ-^{32}P]ATP for 1 h at 37°C. Positive control reactions were performed using a substrate containing an optimised CKII recognition site (-DSDDDDD-). Reactions were stopped by boiling samples in the presence of an equal volume of SDS-PAGE sample buffer, the proteins separated by SDS-PAGE and phosphorylated proteins detected by exposure to X-ray film.

2.4. Fusion protein expression and purification

Wild type or tyrosine 333 to alanine mutant TGN38 cytosolic domains fused to glutathione *S*-transferase (GST-TGN38wt and GST-Y333A) and GST were expressed and purified as previously described [11]. N-terminally truncated μ2 fused to thioredoxin (TRX-μ2ΔN) was expressed in *Escherichia coli* strain BLR(DE3) (Invitrogen) containing the pET32-μ2ΔN plasmid (previously described in [11]) as follows. A saturated overnight culture was diluted 1:100 into 500 ml of Luria-Bertani broth (10 g l^{-1} tryptone (Difco Laboratories, West Mosely, UK), 5 g l^{-1} yeast extract (Difco), 5 g l^{-1} NaCl+50 μg ml^{-1} ampicillin) and grown to saturation overnight at 37°C, 220 rpm. Fusion protein production was induced by incubating the 500 ml saturated culture in the presence of 1 mM IPTG for 1–2 h at 37°C. The cells were then harvested by centrifugation (4800 rpm, 20 min, 4°C, HF6000A rotor (Sorvall)). Harvested cells were resuspended in 40 ml ice cold Tris buffered saline (TBS) supplemented with protease inhibitors (1 μg ml^{-1} leupeptin, 2 μg ml^{-1} antipain, 10 μg ml^{-1} benzamidine, 10 units ml^{-1} aprotinin, 1 μg ml^{-1} chymostatin, 1 μg ml^{-1} pepstatin A, 1 mM PMSF) and lysed by sonication. Cell debris was removed by centrifugation (18 000 rpm, 30 min, 4°C, SS-34 rotor) and TRX-μ2ΔN purified from the supernatant using Talon His-tag affinity resin (Clontech) following the manufacturer's instructions.

2.5. Far-Western assay of interaction between TGN38 cytosolic domain and phosphorylated μ2

5 μg of GST-TGN38wt, GST-Y333A or GST and 10 μl of 1:25 or 1:100 diluted monoclonal anti-thioredoxin antibody (Invitrogen) were immobilised on PVDF (DuPont NEN Ltd, Stevenage, UK) membrane strips by slot blotting and membranes were incubated in three changes of blocking buffer (Tris-buffered saline, 10% w/v dried milk powder). TRX-μ2ΔN was phosphorylated by the clathrin coated vesicle associated kinase activity as described above and purified using Talon His-tag affinity resin (Clontech) following the manufacturer's instructions. Membrane strips were incubated with varying concentrations of phosphorylated TRX-μ2ΔN (2.5–50 μg ml^{-1}) in blocking buffer for 2–3 h at room temperature and washed several times with TBS. Membranes were dried and the presence of radioactivity detected with a Molecular Dynamics Phosphor-imager. Radioactive signals were quantified using the Imagequant software (version 3.3, Molecular Dynamics). Identical experiments were performed with non-phosphorylated TRX-μ2ΔN where the TRX-μ2ΔN was subjected to the same treatments except for the absence of clathrin coated vesicle associated kinase and [γ-^{32}P]ATP. Membranes were incubated with non-phosphorylated TRX-μ2ΔN in blocking buffer for 2–3 h, washed three times in TBS and incubated with 1:1000 polyclonal anti-thioredoxin (raised against recombinant thioredoxin, D.J. Stephens and G. Banting, unpublished data) in blocking buffer for 20 min at room temperature. Membranes were then washed three times in TBS, incubated with 1:10 000 anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham International plc, Little Chalfont, UK), washed six times in TBS and developed using a chemiluminescence system (Boehringer Mannheim). Chemiluminescence signals were quantified following scanning of individual films on an Apple Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http:\\rsb.info.nih.gov\nih-image\).

3. Results and discussion

Utilising the methodologies previously described [14,17,18], we have isolated the peripheral proteins associated with clathrin coated vesicles purified from rat brains. This protein preparation has kinase activity and specifically phosphorylates a thioredoxin tagged soluble recombinant fusion protein of N-terminally truncated μ2 (TRX-μ2ΔN) (Fig. 1, lane 5). It is of note that the C-terminal two thirds of μ2, which is present in this recombinant protein (TRX-μ2ΔN), has been reported to be the domain responsible for interaction with tyrosine based

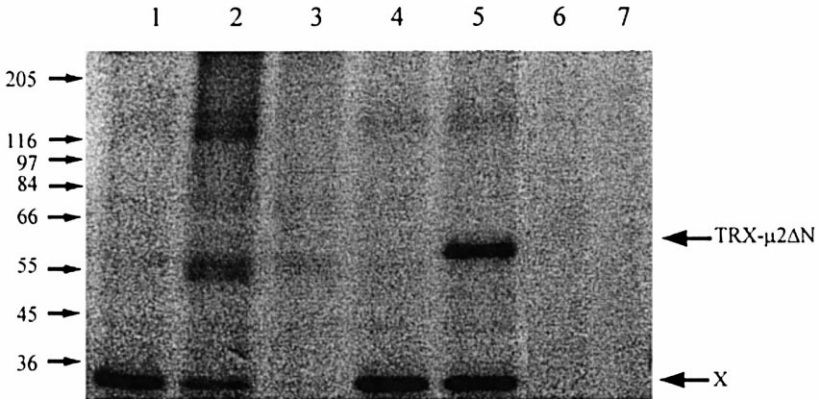

Fig. 1. Phosphorylation of μ2 by clathrin coated vesicle associated kinase activity. Clathrin coated vesicle associated kinase activity was prepared from purified rat brain clathrin coated vesicles as described. The kinase activity was stimulated and the following reactions performed for 30 min at room temperature, followed by separation of proteins by SDS-PAGE and detection of radioactive products by autoradiography. 1: Kinase preparation alone; 2: whole clathrin coated vesicles alone; 3: stripped vesicles alone; 4: TRX-μ1ΔN+kinase preparation; 5: TRX-μ2ΔN+kinase preparation; 6: TRX-μ1ΔN alone; 7: TRX-μ2ΔN alone.

motifs, whereas the N-terminal one third, which is absent from TRX-μ2ΔN, has been reported to be the domain involved in interaction with the rest of the AP-2 adaptor complex [19]. Therefore phosphorylation of μ2 occurs within the domain which interacts with tyrosine based motifs of integral membrane proteins. No phosphorylation of a thioredoxin tagged soluble recombinant fusion protein of N-terminally truncated μ1 (TRX-μ1ΔN) was observed by the clathrin vesicle associated kinase preparation (Fig. 1, lane 4). No phosphorylation of the fusion proteins of either μ chain was observed in the absence of the clathrin coated vesicle associated kinase (Fig. 1, lanes 6 and 7). There is a constitutively phosphorylated protein present upon kinase stimulation (denoted X) that could conceivably be an autophosphorylation event of some activated kinase, or phosphorylation of some other unknown substrate (Fig. 1, lanes 1, 2, 4, 5). The kinase(s) can also be activated while still associated with the clathrin coated vesicles (Fig. 1, lane 2); such preparations contain additional phosphorylation substrates that may be the μ chains or other adaptins already present on the vesicles. Furthermore the vesicles which remain after peripheral proteins have been stripped by treatment with 0.5 M Tris no longer appear to have any kinase activity (Fig. 1, lane 3).

We hypothesised that as phosphorylation of the β adaptins appears to inhibit clathrin-adaptor complex reassociation [14], the concomitant phosphorylation of the μ chains might be a convenient mechanism for also preventing adaptor reassociation with integral membrane proteins. To test this hypothesis we analysed the effect of phosphorylation of μ2 on its interaction with the cytosolic domain of TGN38, an interaction we have previously described [11]. To do this we utilised a far-western blot procedure that we have previously used to show inhibition of the interaction between TGN38 and μ2 by specific tyrphostins [20]. GST fusion proteins of TGN38 cytosolic domains and monoclonal anti-TRX antibodies were immobilised on PVDF membrane filters. These filters were incubated with the TRX-μ2ΔN phosphorylated in the presence of [γ-^{32}P]ATP followed by incubation of the membranes with a polyclonal antibody to TRX and subsequent alkaline phosphatase conjugated secondary antibodies. Development of the membranes with chromogenic substrates for alkaline phosphatase showed the total amount of TRX-μ2ΔN interacting with each immobilised protein (Fig. 2A). Exposure of membranes to a Phosphor-imager screen and subsequent analysis showed relative amounts of phosphorylated TRX-μ2ΔN interacting with each immobilised protein (Fig. 2B). The presence of the immobilised monoclonal antibodies to TRX on the PVDF membranes serves as a positive control for the interaction, allowing a mechanism for immobilising the TRX-μ2ΔN protein irrespective of the μ2 domain or its phosphorylation (Fig. 2A,B, positions 4 and 5). The use of monoclonal antibodies in this manner should not interfere with the detection of total interaction levels using polyclonal anti-TRX antibodies due to differing epitopes being recognised by the antibodies. As can be seen, phosphorylated μ2 can interact with TGN38 wild type cytosolic domain (Fig. 2B, position 1). There is no interaction of μ2 (phosphorylated or otherwise) with TGN38 cytosolic domain in which the tyrosine of the YQRL motif has been mutated to alanine (Y333A mutant) or with GST alone (Fig. 2A,B, positions 2 and 3 respectively). Fig. 2C shows a histogram representation of the relative levels of interaction of phosphorylated μ2 with wild type or Y333A mutant TGN38 cytosolic domain, or with GST. These data suggest that μ chain phosphorylation by clathrin coated vesicle associated kinase(s) does not prevent interaction with tyrosine motif containing integral membrane proteins.

To determine if phosphorylation of μ2 was having a more subtle effect on interaction with TGN38, reducing the affinity of the interaction rather than abolishing it, the above experiment was repeated using a concentration range of μ2. Parallel experiments were performed in which PVDF membrane filters with immobilised TGN38wt and monoclonal anti-TRX antibody dilutions were incubated with a concentration range of phosphorylated or non-phosphorylated μ2. Interaction levels were detected by Phosphor-imager analysis, or polyclonal anti-TRX followed by horseradish peroxidase conjugated secondary antibodies and subsequent chemiluminescence detection. Interaction levels were then quantitated using either the Molecular Dynamics Imagequant program for the Phosphor-imager data, or the public domain NIH Image program for the chemiluminescence data, and curves plotted for interaction of μ2 (± phosphorylation) with TGN38wt and 1:100 monoclonal anti-TRX. As can be seen from Fig. 3 the curves for interaction with TGN38wt and anti-TRX are very similar

for non-phosphorylated μ2 (Fig. 3A). Furthermore the curves for interaction of phosphorylated μ2 with both TGN38wt and anti-TRX are also very similar (Fig. 3B). It may be noted that the interaction levels for phosphorylated μ2 do not reach saturation within the concentration range used in the experiment, whereas interaction levels for non-phosphorylated μ2 do reach saturation. This is likely to be due to competition by non-phosphorylated μ2 present within the phosphorylated μ2 binding assay because, in our hands, the phosphorylation reaction never reached a 1:1 stoichiometry of phosphate to μ2 protein. However, whether or not saturation is reached, these data suggest that this interaction is largely unaffected by phosphorylation of μ2 because the binding curves for the μ2-TGN38 interaction closely match the control (antibody) binding curves irrespective of the phosphorylation state of μ2. Therefore these data strengthen the argument that there is no significant effect of phosphorylation of μ2 by clathrin coated vesicle associated kinase activity upon the interaction with the cytosolic domain of TGN38 in vitro.

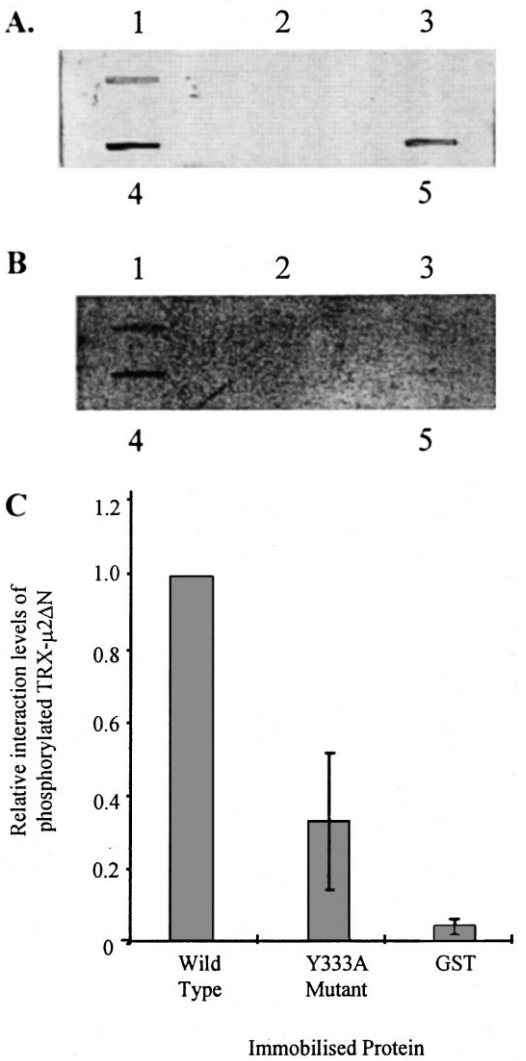

Fig. 2. Interaction of phosphorylated μ2 with TGN38 cytosolic domain. PVDF membrane strips with immobilised GST fusion proteins and monoclonal anti-TRX antibody dilutions were incubated with phosphorylated TRX-μ2ΔN as described. Interaction of total TRX-μ2ΔN was detected by incubation with polyclonal antibodies to TRX, followed by alkaline phosphatase conjugated secondary antibodies and colorimetric detection (A), whereas interaction of phosphorylated TRX-μ2ΔN was detected by Phosphor-imager analysis (B). Immobilised proteins are: 1: GST-TGN38wt; 2: GST-Y333A; 3: GST; 4: 1:25 monoclonal anti-TRX; 5: 1:100 monoclonal anti-TRX. Relative levels of interaction of phosphorylated TRX-μ2ΔN with GST-TGN38wt, GST-Y333A and GST are represented in histogram form (C), data are mean ± S.D. from three independent experiments.

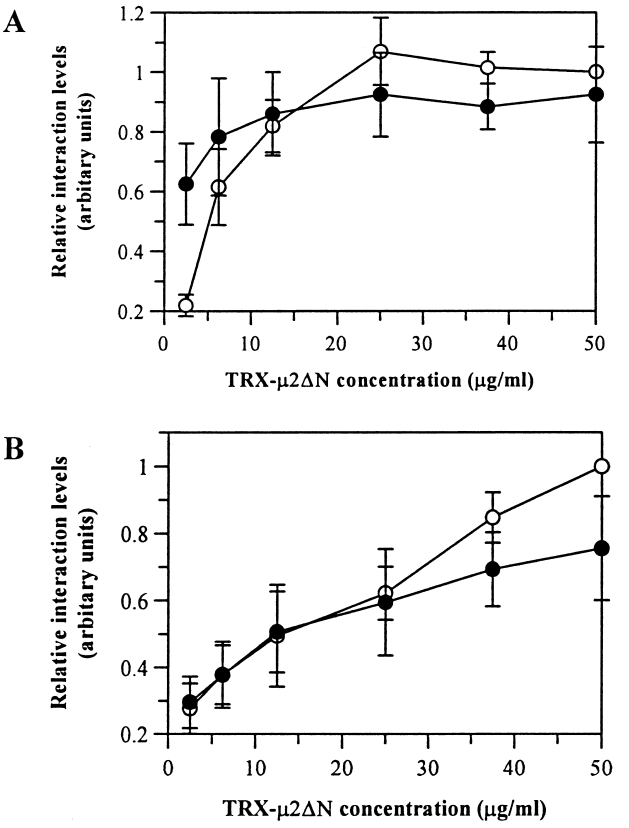

Fig. 3. Interaction of phosphorylated μ2 with TGN38 cytosolic domain over a concentration range of μ2. PVDF membrane strips with immobilised GST-TGN38wt and monoclonal anti-TRX antibody dilutions were incubated with varying concentrations of phosphorylated or non-phosphorylated TRX-μ2ΔN as described. Interaction of phosphorylated TRX-μ2ΔN was detected by Phosphor-imager analysis and interaction of non-phosphorylated TRX-μ2ΔN detected by incubation with polyclonal anti-TRX antibodies followed by horseradish peroxidase conjugated secondary antibodies and chemiluminescence detection. Interaction levels were quantified using the imagequant program for Phosphor-imager data and the public domain NIH image analysis program for chemiluminescence data. Results were plotted for comparison of interactions of non-phosphorylated (A) and phosphorylated (B) TRX-μ2ΔN with TGN38wt (○) and 1:100 monoclonal anti-TRX (●). Data are mean ± S.D. from three independent experiments.

These results were unexpected given the fact that phosphorylation appears to regulate the association and disassociation of various mediators of clathrin coated vesicle formation. Data have shown that the phosphorylation of β adaptin subunits inhibits the association of adaptors with clathrin, probably through phosphorylation at the hinge domain of the β adaptins [14]. Furthermore recent data have shown that additional mediators of clathrin coated vesicle biogenesis including dynamin 1, synaptojanin and amphiphysin 1 are targets for phosphorylation [16]. Dynamin 1 is a GTPase which oligomerises at the neck of deeply invaginated clathrin coated pits

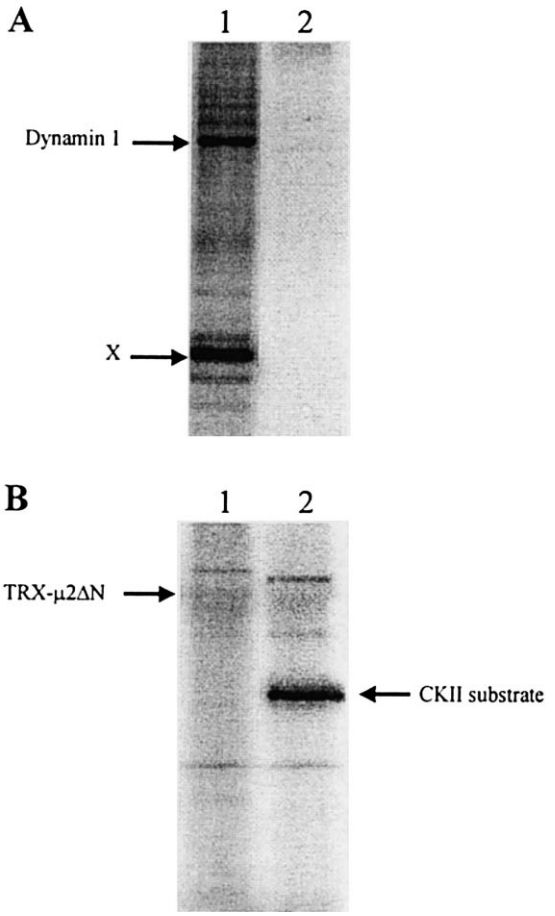

Fig. 4. Phosphorylation of μ2 by casein kinase II and dynamin 1 by clathrin coated vesicle associated kinase. A: Dynamin 1 was incubated with and without clathrin coated vesicle associated kinase under phosphorylating conditions as described, separated by SDS-PAGE and detected by autoradiography. 1: Dynamin 1+kinase preparation; 2: dynamin 1+no kinase preparation. B: Proteins were phosphorylated with casein kinase II as described, separated by SDS-PAGE and detected by autoradiography. 1: TRX-μ2ΔN+kinase; 2: control casein kinase II substrate+kinase.

and is thought to be essential for the fission of clathrin coated vesicles (reviewed in [21]). Synaptojanin is an inositol 5-phosphatase found enriched on synaptic endocytic intermediates [22]. Amphiphysin 1 forms a heterodimeric complex with amphiphysin 2 and as a dimer binds both dynamin 1 and synaptojanin via SH3 domain interactions and also clathrin and the α adaptin subunit of AP-2 (reviewed in [23]). Disruption of amphiphysin SH3 domain interactions has been shown to block clathrin mediated endocytosis [24,25] and it is thought that amphiphysin plays a role in localising dynamin 1 and synaptojanin to clathrin coated pits. Data have shown that the phosphorylation of dynamin 1 and synaptojanin inhibits their interaction with amphiphysin and phosphorylation of amphiphysin 1 inhibits the interaction of the amphiphysin dimer with clathrin and AP-2 [16]. Dephosphorylation of these components also appears to be a prerequisite to allow these proteins to associate. Therefore numerous components of the clathrin mediated endocytic pathway are targets for phosphorylation and in general phosphorylation appears to be the 'inactive' state whereas dephosphorylation is the 'active' state allowing the clathrin coat to form and endocytosis to proceed. It is interesting to note that the clathrin coated vesicle associated kinase(s) activity isolated from rat brain can also phosphorylate both dynamin 1 (Fig. 4A) and synaptojanin (data not shown) which have been purified from rat brain (dynamin 1 and synaptojanin were generous gifts from Harvey McMahon, LMB Cambridge). It has been reported that a casein kinase II activity associated with clathrin coated vesicles can phosphorylate the LCb clathrin light chain [26]. The preparation of clathrin coated vesicle associated kinase(s) used in this study contains a kinase activity which efficiently phosphorylates a fusion protein containing an optimised casein kinase II recognition site (-DSDDDDD-) (data not shown), demonstrating the presence of a casein kinase II like activity. However, recombinant casein kinase II does not phosphorylate TRX-μ2ΔN in the presence of ATP (Fig. 4B). Casein kinase II can also use GTP as a phosphate donor but the effect of GTP in the phosphorylation of μ2 by casein kinase II was not determined.

In summary, we have shown that the kinase activity associated with clathrin coated vesicles can specifically phosphorylate a soluble fusion protein of μ2 and that this phosphorylation does not appear to inhibit the in vitro interaction of μ2 with the cytosolic domain of TGN38, a tyrosine motif containing protein. Our data do not rule out an effect of μ2 phosphorylation on interaction of the AP-2 complex with tyrosine motifs in vivo. Furthermore it is conceivable that phosphorylation of μ chains and other adaptor subunits while having no effect on interaction with tyrosine motifs, may affect the recruitment of cytosolic adaptors to membranes. An as yet uncharacterised protein complex has been reported to be involved in recruiting AP-1 to the TGN membrane [27,28] and a similar complex may exist at the plasma membrane for recruitment of AP-2. Therefore phosphorylation of μ chains and/or other adaptor subunits may be a mechanism for regulating this recruitment and would follow the pattern of phosphorylation of clathrin coat components being the mechanism for retaining the complex in a dissociated or 'off' state. The fact that the same kinase preparation from clathrin coated vesicles has activity towards β-adaptins, μ chains, clathrin chains, dynamin 1, and synaptojanin is of interest as phosphorylation of some of these proteins has been shown to prevent interactions between the various clathrin coat components. Although it is yet to be determined if there is more than one kinase activity present associated with clathrin coated vesicles, these data may suggest that the same kinase could be responsible for all these phosphorylations. This could allow a single point for regulation of all these phosphorylation events; a process that may control a regulated association and disassociation cycle of the clathrin mediated endocytosis machinery.

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