Biochemical and Biophysical Research Communications **255,** 54–58 (1999)
Article ID bbrc.1998.0140, available online at http://www.idealibrary.com on IDEAL®

In Vitro Binding Study of Adaptor Protein Complex (AP-1) to Lysosomal Targeting Motif (LI-Motif)

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Received December 11, 1998

Lysosomal membrane glycoproteins carry targeting information in cytoplasmic regions. Two distinct targeting motifs in these regions, GY (glycine-tyrosine) and LI (leucine-isoleucine), have been identified and characterized. Accumulating evidence suggests that the adaptor complexes (AP-1, AP-2, and AP-3) recognize this information in cytoplasmic tails of transmembrane proteins. Here we report two different *in vitro* analyses (affinity beads and surface plasmon resonance) which revealed specific interaction between the cytoplasmic tail of LGP85 and AP-1 but not so with AP-2. We also noted requirement of the LI motif of the LGP85 tail in binding to the AP-1 complex. Our data and others which indicated the binding of AP-3 to the LIMP II (synonym of LGP85) tail suggest that the cytoplasmic tail of LGP85 interacts with AP-1 at the *trans*-Golgi network (TGN) and AP-3 at late endosomes, respectively. We propose that this sequential interaction between the lysosomal targeting signal and distinct APs along its transport pathway is responsible for the critical sorting of lysosomal membrane proteins and/or the potential proofreading system of mistargeted molecules. © 1999 Academic Press

There are two distinct targeting signals of lysosomal membrane proteins, so-called tyrosine based motif and di-leucine based motif (1–12). Both signals are necessary for intracellular sorting of membrane proteins to lysosomes, however, cytoplasmic molecules which recognize these signals have remained to be identified. There are numerous coats for vesicle formation, even from one compartment, and it has been postulated that most of membrane proteins which carry targeting information in the cytoplasmic portions recognized by the coat molecules at the cytoplasmic face of the membranes (13–17).

Clathrin coated vesicles (CCVs) are one of the well characterized coated vesicles and originate from either TGN or plasma membrane (PM) (18, 19). The AP-1 complex is restricted to the CCVs from TGN, whereas the AP-2 complex contributes to the formation of CCVs and clathrin coated pits from PM. It was also reported that CCVs bud from endosomal compartments (20), therefore the involvement of CCVs for vesicle transport steps are more complicated than heretofore considered to be. Recent studies revealed novel adaptor related complexes, AP-3 as a coat for vesicles derived from TGN/endosomes to lysosomes (21–24). Although AP-3 shares structural composition similar to AP-1/AP-2 complexes (heterotetramer), it has no affinity for the clathrin lattice. In the fruit fly (*Drosophila melanogaster*) (22), yeast cells (*Saccharomyces cerevisiae*) (21, 25), and coat color mutation mouse (26), genetic defects in one of AP-3 subunits lead to a significant perturbation in vesicle transport steps after TGN/late endosomal compartments. These genetic evidences and data obtained in immuno-localization studies of AP-3 strongly suggest the involvement of AP-3 in vesicle formation at TGN and/or late endosomes.

LGP85 (LIMP II) has a lysosomal targeting signal, leucine-isoleucine motif, at carboxyl terminal of cytoplasmic domain (7–9, 11, 27). Although recent study indicated the cytoplasmic tail of LIMP II was recognized by AP-3 at endosomal compartment (28), we hypothesized that LGP85 is segregated from secretory pathway at TGN, the primary sorting station for the lysosomal membrane proteins with AP-1 binding prior reaching to the second station, endosomes. Here we tested the *in vitro* AP-1 binding to the LGP85 tail and we propose such specific signal recognition performed by AP-1 is apparently responsible for sorting and transport of some lysosomal membrane proteins at TGN.

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Abbreviations used: TGN, *trans*-Golgi network; AP, adaptor protein; LGP, lysosomal glycoprotein; LAP, lysosomal acid phosphatase; PM, plasma membrane; CCV, clathrin coated vesicle; GST, glutathione *S*-transferase; SPR, surface plasmon resonance.

0006-291X/99 $30.00

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MATERIALS AND METHODS

Reagents and antibodies. Synthetic peptides (LGP85 tail: CRGQGSTDEGTADERAPLIRT, mutated LGP85 tail: CRGQGSTDEGTADERAPAART, control peptide (corresponding to an extracellular loop of cholecystokinin receptor A): QTANMCRFLLPNDVMQQ) were provided by TANA Laboratories, L.C. (Houston, TX). All other reagents and compounds were of the highest purity available and were purchased from commercial sources. Monoclonal antibody against α-adaptin (Clone 100/2) was obtained from SIGMA (Tokyo, Japan). Monoclonal antibody against γ-adaptin (Clone 88) or clathrin-heavy chain (Clone 23) was obtained from Transduction Laboratories (Tokyo, Japan). Anti-μ3 polyclonal (rabbit) antibody (29) was kindly provided by M. S. Robinson (Cambridge, UK).

Plasmid construction and purification of GST fusion proteins in E. coli. Constructs encoding the cytoplasmic domain of LGP85 (amino acids 458–478) were generated by polymerase chain reaction (PCR) with oligonucleotides corresponding to the amino- and carboxyl-terminal regions of cytoplasmic domains. Primers (Hokkaido System Science, Hokkaido, Japan) were designed according to the rat LGP85 cDNA sequence to obtain an *Sma*I site at the 5′ end (5′-AGAGCCCGGGATGTCGAGGACAGGGGTCTAC-3′) and a *Sal*I site at the 3′ end (5′-AGAGGTCGACTTAGGTCCGTATGAGGGGTG-3′). To substitute the LI sequence of the cytoplasmic portion of LGP85 with AA (alanine-alanine), a 3′ end primer was designed as (AGAGGTCGACTTAGGTCCGTGCGGCGGGTG). The PCR fragments were directionally cloned into the corresponding sites of pGEX-KG vector (30). Expression and purification of GST fusion protein in *E. coli* was described elsewhere (31).

Preparation of adaptor complexes from rat liver clathrin-coated vesicles. Clathrin coated vesicles fraction was obtained according to the methods of Campbell *et al* (32) with minor modifications and adaptor proteins were extracted from CCVs according to Sosa *et al* (16).

In vitro binding studies (batch affinity beads analysis). GST or GST-fusion proteins were coupled to CNBr activated Sepharose 4B (Pharmacia) at 1 mg protein/ml beads (33). Prior to use, the beads were blocked with BSA (0.5 mg/ml) for 4–5 h at 4°C. 100 μl of the extracted adaptor proteins fraction (0.38 μg/ml) were incubated with an equal volume of GST or GST-fusion proteins beads, overnight at 4°C, with gentle rotation. The incubated beads were sedimented by brief centrifugation (for 3 min at 750*g*) and then washed 3 times with 750 μl of Mes buffer containing 1 mM Chaps. The washed fractions (2.25 ml) were saved and precipitated by TCA for quantitative immunoblotting. The binding proteins were eluted by boiling with Laemmli's sample buffer containing SDS. Adequate amounts of three fractions were subjected to SDS–PAGE (34) followed by immunoblotting (35) with an antibody to γ-adaptin (AP-1) or α-adaptin (AP-2), respectively, and quantitated by NIH-image. Recoveries of all three fractions varied with the experiment (71–114%).

SPR measurements by IAsys cuvette system. GST and GST-fusion proteins were immobilized on an IAsys cuvette coated with carboxymethyl-dextran according to the manufacturer's instruction. The cuvette was equilibrated with PBS–Tween (0.1%) and the extracted APs were added to a final concentration of 35 μg/ml in a total volume of 150 μl. Changes in the resonant angle were monitored at 1-s intervals for about 300 s, then the cuvette was washed with PBS–Tween to remove non-specific interacting proteins, for the next 300 s. Subsequently, specific antibody against either γ-adaptin or α-adaptin was added to a final concentration of 0.1 mg/ml and responses were monitored for the next 100 s. Experiments were done at room temperature with a stirrer speed of 80 rpm. For peptide competition experiments, APs were preincubated with various amounts of peptide (0.5–62.5 μM) for 1 h on ice before application to the cuvette.

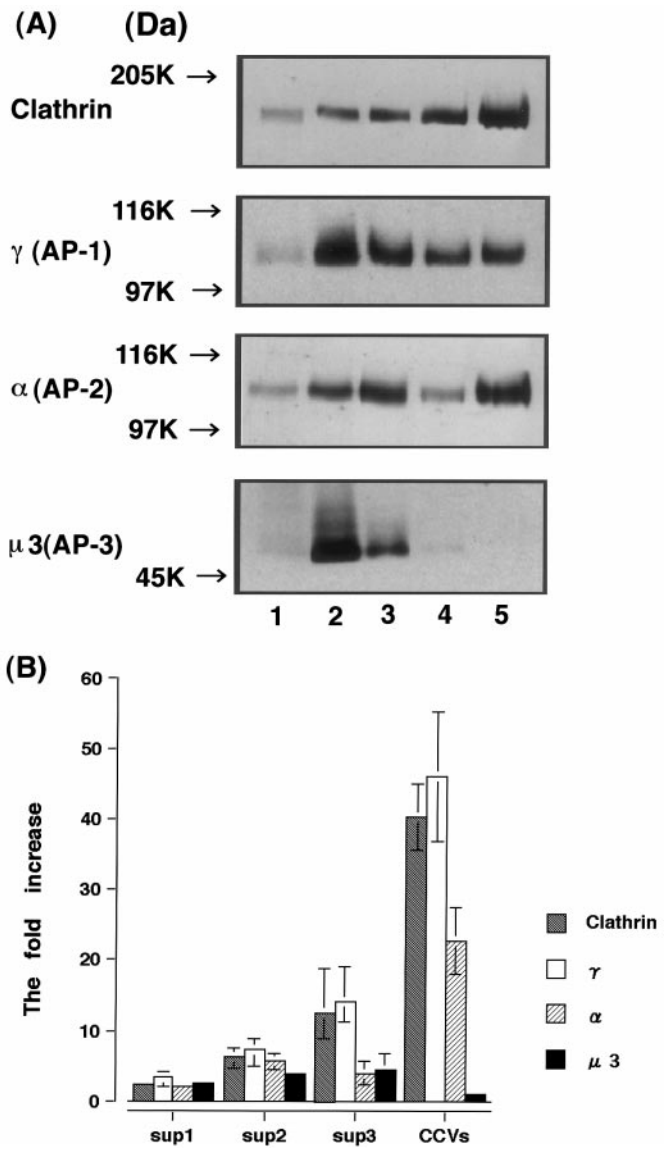

FIG. 1. Subcellular fractionation of clathrin-coated vesicles from rat liver. Fractionated samples were applied to SDS–PAGE and analyzed by immunoblotting with antibody to Clathrin heavy chain, γ-adaptin, α-adaptin and μ3-chain, respectively (A) and quantitated. Lane 1, homogenate; lane 2, 19,000*g* supernatant; lane 3, 43,000*g* pellet; lane 4, 43,000*g* Ficoll/sucrose supernatant; lane 5, clathrin coated vesicle fraction. Enrichments of each fraction (B) are expressed as a fold-increased over the specific activities (a.u./mg protein) to homogenate. Data shown are mean ± SE for $n = 3$.

RESULTS

Subcellular fractionation of clathrin-coated vesicles from rat liver. We prepared CCVs from rat liver (see materials and methods) and quantitative immunoblotting revealed that clathrin heavy chain and γ-adaptin, one of the subunits from the AP-1 complex, were enriched in the final fraction (CCVs) about 40–46 times relative to those in the homogenate (Fig. 1). α-Adaptin, part of the AP-2 complex, was concentrated in the

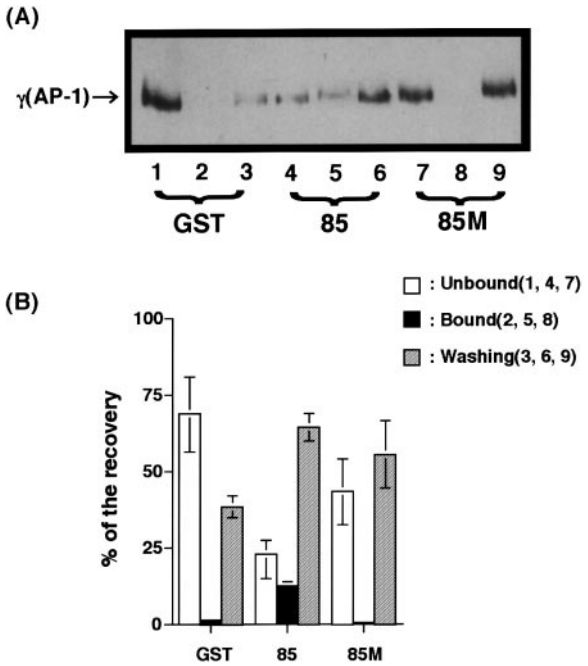

FIG. 2. *In vitro* binding of AP-1 to the affinity beads of GST LGP85 tail. Adaptor proteins (35 μg of protein) were incubated with the affinity beads coupled with GST, GST-LGP85tail (85) or GST-LGP85mutant tail (85M), respectively. After the incubation, three fractions, unbound; lanes 1, 4, 7, bound; lanes 2, 5, 8, and washing; lanes 3, 6, 9, were applied to SDS–PAGE and analyzed by immunoblotting with anti γ-adaptin (for AP-1; A) and quantitated (B). B was expressed as a percentage of the total APs added to the binding assay, and these values are the mean ± SE for $n = 3–4$.

CCVs as well, albeit to a slightly lower extent (about 23 times over homogenate) than those of clathrin and AP-1. Although a fair amount of the μ3 subunit, a medium chain of the AP-3 complex, was expressed in the rat liver, it was hardly detectable in the CCVs (Fig. 1; AP-3). Since AP-3 is a novel coat protein which does not bind to the clathrin lattice and may locate in late endosomes, our prepared CCVs showed high homogeneity in terms of clathrin-coat and did not contain AP-3 coated vesicles. Recoveries of clathrin, γ-adaptin and α-adaptin in the CCVs were 7.9, 9.0, and 4.7%, respectively (data not shown). AP complexes were effectively (nearly 90%, data not shown) extracted from rat liver CCVs with 0.5 M Tris–HCl buffer and dialyzed against MES-buffer for further *in vitro* binding experiments.

In vitro reconstituted binding of AP-1 complexes to the cytoplasmic portions of LGP85. To analyze the *in vitro* interaction between AP-1 and LGP85 tail, we developed the batch analysis method using affinity beads coupled with various GST fusion proteins (see materials and methods). An adequate volume of three fractions, unbound (sup), washing and elution (beads) were subjected to SDS–PAGE followed by immunoblotting with the antibody against γ-adaptin (for AP-1; Fig. 2A) or α-adaptin (for AP-2; data not shown). Quantitative immunoblotting results (Fig. 2B) revealed that AP-1 complex bound to GST-LGP85 tail specifically but not to the GST alone or GST-LAP tail (data not shown). About 10–13% of total AP-1 was recovered in the GST-LGP85 tail beads fraction. The significant redistribution of AP-1 from the unbound fraction to the washing fraction in the GST-LGP85 tail expressed the existence of a weak but specific interaction between AP-1 and LGP85 tail. Disruption of the LI motif in the cytoplasmic portion of LGP85 led to loss of AP-1 binding to the beads, a finding indicating that this interaction was in an LI motif dependent manner. Interestingly, no AP-2 binding to GST-LGP85 tail beads was observed (data not shown). The recoveries of AP-1 and AP-2 from all three fractions varied with the experiment but ranges were reasonably small (between 71 and 114%).

SPR measurements for binding of AP-1 to the cytoplasmic tail of LGP85. We monitored real time binding of APs to the cuvette which had been coupled with GST-LGP85 tail up to 300 s, then washed the cuvette with PBS–Tween (see Materials and Methods). The washing significantly reduced signals from the nonspecific interaction but retained the specific response between APs and GST-LGP85 tail. Subsequently, we added a specific antibody to each subunit of APs (α-adaptin or γ-adaptin, respectively) and followed binding of the antibody to APs on the cuvette, through GST-LGP85 tail. We noted that signals derived from interaction with AP-1 but not with AP-2 (Fig. 3). The substitution of LI with AA significantly decreased the response to APs (about 70% of LI-motif's one) and abolished the following response to the AP-1 antibody (Fig. 4). These data were consistent with data obtained from affinity beads analysis (Fig. 2). Preincubation of APs fraction with LGP85 tail peptide inhibited the interaction with GST-LGP85 tail, in a concentration dependent manner (Fig. 5). Over 12.5 μM LGP85 tail peptide inhibited AP-1 binding to the cuvette, 2.5 μM peptide showed about 30% of maximum binding, while less than 0.5 μM peptide had no apparent effect on the binding. Control peptide (Fig. 5) and mutated LGP85 tail peptide (data not shown) had no significant effect on the response at the maximum concentration (62.5 μM), which meant that this interaction was specific and LI-motif dependent. By using same SPR measurement, we also observed the specific interaction between GST-LAP tail and AP-2 but not with AP-1 as previously reported by different methods and the specificity of these interaction was confirmed by the peptide competition as shown in Fig. 5 (data not shown).

DISCUSSION

LGP85 (7, 10), also known as LIMP II (8, 9), is one of the major lysosomal membrane glycoproteins and is delivered to lysosomes LI-motif dependently (11, 27),

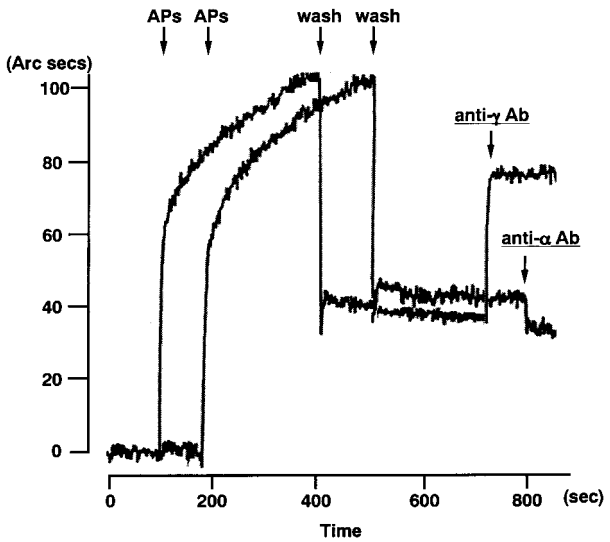

FIG. 3. Analysis of APs (AP-1/AP-2) binding to the immobilized GST-LGP85 tail by IAsys. GST-LGP85tail was immobilized on a carboxymethyl dextran cuvette surface (see Materials and Methods). Changes in resonant angle were monitored continuously for 5 min following the addition of adaptor proteins (final 35 μg/ml in PBS–Tween buffer). Subsequently the unbound materials were washed-out with PBS–Tween buffer and then the bound APs were probed with either anti γ-adaptin (for AP-1) or α-adaptin (for AP-2) antibody (0.1 mg/ml). GST-cuvette was analyzed by the same way as control (data not shown).

however the synthetic route of LGP85 has heretofore not been characterized. We have shown here that specific binding of the LGP85 tail to AP-1 provides related

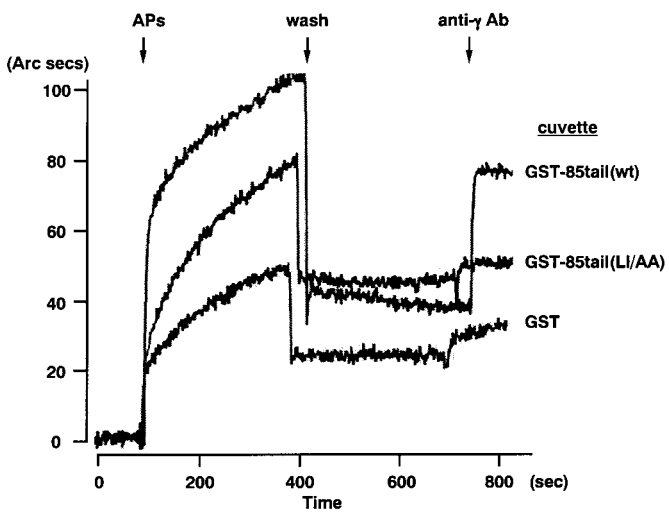

FIG. 4. Binding analysis of AP-1 to the immobilized GST-LGP85 tail mutant (LI/AA) by IAsys. GST, GST-LGP85tail and GST-LGP85 mutant were immobilized on the cuvette surface, respectively. Changes in resonant angle were monitored continuously for 5 min following the addition of adaptor proteins (final 35 μg/ml in PBS–Tween buffer). Subsequently the unbound materials were washed-out with PBS–Tween buffer and then the bound AP-1 was probed with anti γ-adaptin antibody (0.1 mg/ml).

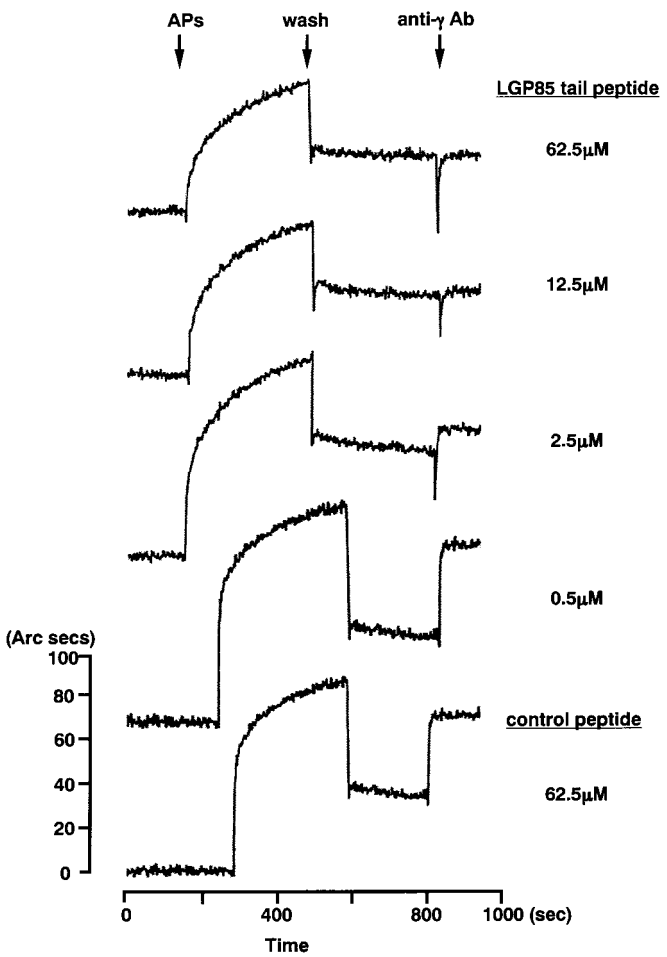

FIG. 5. Peptide competition of AP-1 binding to the immobilized GST-LGP85 tail. Adaptor proteins (15 μl) were preincubated in the presence of the synthetic LGP85 tail peptide or control peptide with the indicated concentrations for 1 hr and then applied to the cuvette which is immobilized GST-LGP85 tail. Subsequently the unbound materials were washed out with PBS–Tween buffer and then the bound adaptor proteins were probed with anti γ-adaptin antibody (0.1 mg/ml).

clues as to how lysosomal membrane proteins are sorted and delivered to lysosomes. We used two different methods, the affinity beads and SPR measurements for *in vitro* binding analysis. Both analysis revealed specific interactions between LGP85 tail and AP-1, in a LI-motif dependent manner. This seems to be the first observation of AP-1 binding to the LGP85 cytoplasmic tail and suggests that transport steps of LGP85 to lysosomes involves a clathrin/AP-1 mediated budding from TGN.

Based on our data and others (28), we proposed that sequential interaction of AP-complexes (AP-1 and AP-3) with LGP85 tail facilitated their targeting routes to lysosomes. The specific interaction of AP-1 with the LGP85 tail suggests that LGP85 is packed into CCVs at TGN and then transported to late endosome. Subsequently LGP85 is sorted from recycling receptors

such as mannose-6-phosphate receptor (36) at late endosomes by the interaction with AP-3 and then delivered to lysosomes. We propose that this two steps sorting mechanism might be required for the direct targeting of LGP85 to lysosomes. On the other hand, LAP is exiting TGN by the bulk flow because of no interaction of its tail with AP-1 at TGN and once LAP reaches the PM, the cytoplasmic tail is recognized by AP-2 and selectively enriched into CCV (16, 37). Then LAP takes the endocytic route to lysosomes. Recently it has been reported the binding of LL-motif to β-subunit of AP-1 complex (38). It remains to be elucidated which subunit of AP-1 interact with LGP85 tail. Further analysis, such as yeast two hybrid systems and photocross linking are needed to elucidate the answer.

ACKNOWLEDGMENTS

We thank Dr. M. S. Robinson for kindly providing anti-μ3 antibody, M. Tsurumaru for helpful assistance and discussion on SPR measurements, and M. Ohara for comments on the manuscript. This work was supported in part by CREST grant from the Sciences and Technology Corporation of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

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