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IDENTIFICATION OF THE CLATHRIN-BINDING DOMAIN OF ASSEMBLY PROTEIN AP-2

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The clathrin binding domain of the assembly protein AP-2 has been identified by proteolytically cleaving AP-2 into 2 discrete moieties, termed light and heavy mero-AP (LM-AP and HM-AP), and testing their ability to bind to clathrin assembled into cage structures or to clathrin trimers immobilized on Sepharose. The smaller product (LM-AP), which contains 20-40-kD fragments of the parent 100-kD polypeptides and which comprises two small appendages in the native AP-2 molecule, did not significantly interact with clathrin under either condition. In contrast, the HM-AP complex, which forms the larger central mass of the native AP-2 structure and contains uncleaved 50-kD and 16-kD polypeptides as well as 60-66-kD fragments of the parent 100-kD polypeptides, retained binding activity for both dissociated and assembled clathrin.

The clathrin coated membrane is implicated in various receptor-mediated processes of membrane dynamics, particularly endocytosis (1) and Golgi function (2). The characteristic covering of the coated membrane is composed of two major kinds of protein components: clathrin, a triskelion shaped protein responsible for the polygonal latticework of the coat (3); and members of a family of proteins that have been referred to as assembly proteins or AP's because they promote the formation of complete coats from dissociated clathrin under physiological conditions (4). The major assembly protein of bovine brain coated vesicles, designated AP-2, has recently been purified and characterized by several different laboratories and found to be a complex composed of 100-115-kD (AP100), 50-kD (AP50) and 16-kD polypeptides (5-7). Analysis of the AP-2 by freeze-etch electron microscopy revealed a tripartite structure composed of a large central brick-like mass and two smaller, symmetrically displaced appendages (8). Utilizing earlier proteolysis studies (9), this report also showed that the central brick contained the intact 50- and

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16-kD polypeptides and approximately 60-66-kD of the AP100. The appendages, which are released on proteolysis, contain the remainder of the AP100 and are 28-40-kD in size depending on the protease used.

AP binds directly to clathrin, manifested by its stoichiometric incorporation into the assembling coat structures (10). Data has also been presented suggesting that AP also binds to receptors that undergo endocytosis (11). This biochemical bifunctionality would be consistent with the concept that, in situ, the AP molecule bridges the distance between the latticework of the clathrin shell and the enclosed vesicle membrane, presumably interacting with both (8, 12). In this context, it is important to determine which of the domains of the AP-2 are involved in recognition and binding of clathrin. In this report we show that it is the central brick-like mass of the AP, containing the intact 16- and 50-kD polypeptides and the 60-66-kD moiety of the AP100, that retains the ability to recognize clathrin. In light of this assignment of function to a part of the parent protein, and the clear compositional and morphological differences between the two domains, we term the larger, clathrin-binding domain "heavy mero-AP" (HM-AP), and its smaller counterpart "light mero-AP" (LM-AP).

MATERIALS AND METHODS

MATERIALS: Clathrin-Sepharose and Superose gel filtration-purified clathrin and AP were prepared as described previously (5). Reassembled clathrin cages were prepared by dialyzing clathrin overnight at 4° against 5mM sodium MES - 2 mM $CaCl_2$, pH 6.10, and were collected by centrifugation at 75,000 rpm for 7 min at 4° in a TLA 100.2 rotor (Beckman). TPCK-treated trypsin was from Worthington Biochemical; elastase and soybean trypsin inhibitor were from Sigma. All other chemicals used were reagent grade or better.

METHODS: Mero-AP preparations (in 0.5M Tris-HCl, pH 7) were generated by treating AP at 23° with elastase (500:1, by weight) for 60 min followed by a 1.5-fold molar excess of alpha-2-macroglobulin; or with trypsin (200:1, by weight) for 30 min followed by a 1.5-fold molar excess of trypsin inhibitor. AP and mero-AP (0.56 mg) binding to clathrin-Sepharose (1 ml containing 1.3 mg clathrin) was performed as described previously (5). After overnight dialysis against buffer A (0.1 M MES, 1mM EGTA, 0.5 mM $MgCl_2$, 0.02% NaN_3, pH 6.5), the resin was washed by resuspension with 1 ml aliquots of buffer A and then eluted with 0.5 M Tris-HCl, pH 7. Fractions were precipitated with 10% TCA, resuspended, and equal portions subjected to electrophoresis on 5-8.5% polyacrylamide gradient gels (5). For cage binding assays, clathrin cages (60 ug) that had been centrifuged immediately before use were incubated for 90 min at 4° with AP or mero-AP (60 ug) in a final volume of 0.8 ml of 0.1 M sodium MES 50mM Tris-HCl, pH 6.5. After a brief (5 min) spin at 10,000 xg, cages with bound AP/mero-AP were collected by centrifugation at 75,000 rpm

as above. Equal portions of the supernatants and pellets were collected and applied to an 8.5% polyacrylamide minigel (Bio-Rad). Densitometry was performed with a Hoefer GS-300 densitometer using GS-360 data analysis software: scans of paired supernatant and pellet lanes were performed with equal gain and amplification settings.

RESULTS

We have previously shown that AP-2 will bind to clathrin trimers immobilized on a Sepharose resin and that the technique can be used to purify AP-2 from a partially purified assembly protein preparation obtained by gel filtration (5). As shown in Fig. 1, when clathrin-Sepharose is incubated with excess AP, some AP as well as contaminants are present in the flow through of the column (Fig. 1, lane 1). Subsequently, stripping of the resin with 0.5M Tris-HCl, which breaks the non-covalent clathrin-AP bond (5), results in the elution of the purified AP-2 complex containing ~100-115-kD polypeptides, 50-kD and 16-kD polypeptides (Fig. 1, lane 2).

A similar experiment was performed with elastase-treated AP which contained both heavy and light mero-AP (HM-AP and LM-AP, respectively; see Introduction). A portion of the elastase-treated AP preparation did bind to the clathrin-Sepharose resin, for on Tris-HCl elution only the HM-AP was found (Fig. 1, lane 4), distinguished by its complement of ~60-66-kD polypeptides derived from the parent 100-kD polypeptides, as well as the uncleaved 50- and 16-kD polypeptides with which they are complexed. In contrast, the LM-AP bands of ~30-kD were found only in the unbound flow through of the column, along with excess HM-AP (Fig. 1, lane 3). These results indicate that the LM-AP does not bind to immobilized clathrin trimers under these conditions but that the HM-AP domain retains the clathrin recognition activity of the parent complex.

To evaluate the binding of AP by assembled clathrin in the form of coat structures, we have developed a novel cage binding assay that will be described in detail elsewhere (Keen, Beck, Kirchhausen and Jarrett, manuscript in preparation). Briefly, free AP is added to preassembled clathrin cages, the mixture is centrifuged, and cages with bound AP are separated from unbound protein in the supernatant. When intact AP (Fig. 2, lane 1) is incubated with

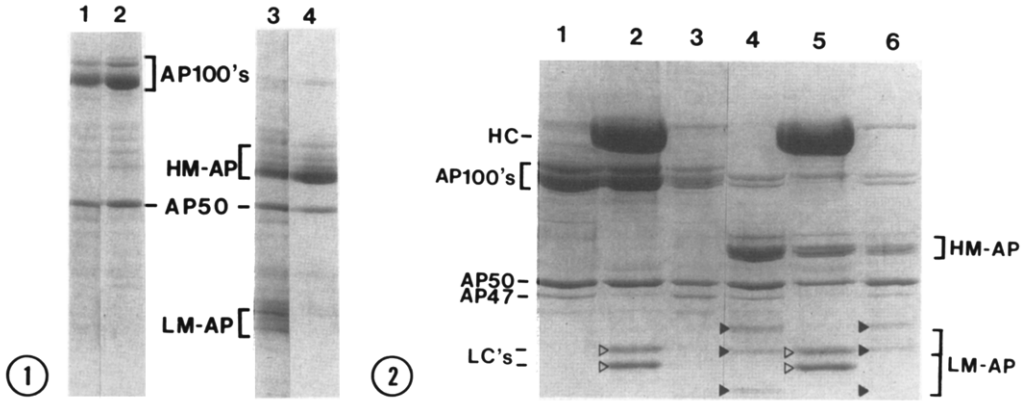

FIG. 1: Binding of AP and mero-AP to clathrin-Sepharose. AP (lanes 1 and 2) and mero-AP (lanes 3 and 4) were incubated with clathrin-Sepharose and unbound protein eluted with buffer A (lanes 1 and 3). Bound protein was then eluted with 0.5M Tris-HCl, pH 7.0 (lanes 2 and 4). HM-AP bracket refers to the region containing the 60-66-kD polypeptide components of the heavy mero-AP complex; LM-AP bracket denotes the region containing the ~30-kD light mero-AP polypeptides that are not retained by the column.

FIG. 2: Binding of AP and mero-AP to clathrin cages. AP (lane 1) or mero-AP (lane 4) was incubated with clathrin cages as described in Methods. The clathrin cages, containing clathrin heavy (HC) and light chains (LC's; open triangles) and cage-bound AP (lane 2) or mero-AP (lane 5), were separated by centrifugation from unbound material (lanes 3 and 6). HM-AP bracket refers to the 60-66-kD polypeptides of the heavy mero-AP complex, while the LM-AP bracket denotes the position of the 40-, 36- and 30-kD light mero-AP polypeptides (solid triangles). Note that the larger of the two clathrin light chains found in the cage pellet comigrates with the 36-kD LM-AP polypeptide; for this reason the proportion of cage-associated LM-AP was determined specifically by densitometry of the 40-kD LM-AP polypeptide (Table 1).

clathrin cages in this way, the pelleted clathrin cages, which contain the clathrin heavy chain (HC) and two clathrin light chains (LC's), are accompanied by cage-bound AP-2 polypeptides (Fig. 2, lane 2). Analysis of the supernatant (Fig. 2, lane 3) reveals a small amount (7%; Table 1) of clathrin as well as some AP that failed to be bound to the cages. In addition, a 47-kD polypeptide that we have previously shown to be a component of the more minor AP-1 complex, and which binds more weakly to clathrin-Sepharose (5), is also present in the supernatant. In the absence of clathrin, a negligible amount of AP is sedimented (not shown).

Mero-AP generated by trypsin treatment is shown in Fig. 2, lane 4. This preparation contains major 60-62-kD and 66-kD polypeptides similar in migration to those produced by elastase treatment (Fig. 1, lane 4), but the LM-AP polypeptides migrate more slowly than their elastase counterparts, with

TABLE 1
BINDING OF INTACT AND MERO-AP TO CLATHRIN CAGES

	INTACT AP		MERO-AP	
	PROTEIN (%) IN		PROTEIN (%) IN	
	CAGE PELLET	SUPT.	CAGE PELLET	SUPT.
Clathrin heavy chain	93	7	96	4
AP100-115	70	30	ND	ND
AP50	67	33	35	65
60-66-kD polypeptides of HM-AP	--	--	55	45
LM-AP (40-kD*)	--	--	19	81

*Only the 40-kD band of the LM-AP could be quantitated because the more rapidly migrating LM-AP band could not be distinguished from clathrin light chain LCa.
ND - Not determined.

apparent molecular weights of 36- and 40-kD (doublet) as well as a smaller band at about 30-kD. Densitometry indicated that the 100-kD polypeptides were more than 85% cleaved. After incubation of this preparation with clathrin cages and sedimentation, the 60-66-kD HM-AP polypeptides were partitioned away from the LM-AP, a majority of the former being found in the cage-containing pellet fraction (Fig. 2, lane 5). In contrast, the supernatant (Fig. 2, lane 6) contained the majority of the LM-AP as indicated by the presence of the 40- and 36-kD LM-AP polypeptides, the minor 30-kD polypeptide being more difficult to detect (marked by solid triangles in Fig. 2, lanes 4 and 6). Quantitation of the reaction (Table 1) was obtained by densitometry, confirming the impression that the LM-AP were retained predominantly in the supernatant (also see Discussion). Note that because the 36-kD LM-AP polypeptide comigrates with the cage-associated clathrin light chain LCa (marked by open triangles in Fig. 2, lanes 2 and 5), quantitation of the relative proportion of cage-bound LM-AP was performed by densitometry of the 40-kD LM-AP polypeptide in lanes 5 and 6.

DISCUSSION

We have previously shown that proteolytic cleavage of the AP100 polypeptides quantitatively abolishes the assembly activity of the complex (9). Here

we show that the larger of the two cleaved products retains it ability to recognize and bind to clathrin, whether the clathrin is present as a free triskelion or in its assembled form in a cage structure. The active binding fragment corresponds to the larger central brick domain of the parent molecule that has been revealed by quick-freeze deep-etch microscopy (8). In view of the correspondence between morphological domain and function, and by analogy to myosin organization (13), the terms heavy and light mero-AP are employed to describe these moieties.

Since isolated 100-kD polypeptides have been shown to be capable of promoting cage assembly (14,15), we can conclude that it is specifically the 60-62/66-kD components of the HM-AP complex that are active in clathrin binding; cleavage of AP within coat structures yields results consistent with this analysis (Kirchhausen, Nathanson, Matsui, Vaisberg, Chow, Burne, Keen and Davis, submitted). Nonetheless, for retention of the cage assembly activity, the integrity of the linkage between the HM-AP and LM-AP is apparently required (9).

Quantitative analysis of the data presented in this report (Table 1) raise two other points. Somewhat less HM-AP than native AP is bound to a constant amount of clathrin cages under the conditions of the experiment, possibly reflecting a change in affinity on proteolysis. In addition, the amount of AP50 bound to cages does not precisely parallel the amount of 60-66-kD HM-AP polypeptides bound, as it does binding of the AP100's in the native complex; a similar effect is qualitatively apparent in binding to free clathrin triskelions (Fig. 1). Apparently, AP50 can dissociate from the AP complex, a phenomenon that may explain differences in AP50/AP100 ratios in AP-2 preparations reported by different labs (5,6,16,17).

If the role of the HM-AP is to bind clathrin and facilitate lattice assembly, what function might the LM-AP fulfill? In view of the recent report that AP-2 binds directly to receptors (11), we suggest that it is the LM-AP that mediates this process. The brick-like hubs of AP-2 molecules (the HM-AP) have been observed to be attached to vesicles by a slender stalk (reference 8,

Fig. 9), suggesting that the smaller LM-AP appendages may anchor the AP-2 molecules to the membrane by interacting with membrane-bound receptors or binding sites (6). Further work will be necessary to test this possibility.

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