Identification of the Phosphorylation Sites of Clathrin Light Chain LCb*

(Received for publication, January 12, 1988)

Beth L. Hilt, Kurt Drickamer, Frances M. Brodsky†, and Peter Parham‡

From the †Department of Cell Biology, Stanford University, Stanford, California 94305, the §Department of Biochemistry, Columbia University, New York, New York 10032, and the ¶Department of Pharmacy, University of California at San Francisco, San Francisco, California 94143

Clathrin light chains, LCa and LCb, are products of two closely related genes whose mRNAs undergo differential splicing to result in at least four different light chain isoforms. The physiological significance of clathrin light chain diversity remains unclear. To date, the only evidence for a functional distinction of LCa and LCb is the preferential phosphorylation of LCb, which takes place at serine residues and is mediated by coated vesicle-associated casein kinase II. As a first step toward determining the function of light chain diversity, we have mapped the in vitro phosphorylation sites on LCb. We use [γ-32P]ATP to phosphorylate LCb within coated vesicles, followed by sequencing of 32P-labeled chymotryptic peptides thereof, to identify serine residues at positions 11 and 13 as the phosphorylation sites. We find that phosphorylation of LCb within coated vesicles can be inhibited by four monoclonal antibodies specific for different epitopes of the clathrin light chains.

Clathrin is the principal protein component of the coat of coated pits and vesicles, structures which regulate receptor-mediated endocytosis and intracellular transport between membrane-bound compartments in eukaryotic cells (1). The unit structure of the clathrin coat is the triskelion which contains three heavy and three light chains of two types, LCa and LCb (2). Investigations into the assembly of clathrin in vitro have suggested that the light chains function in a structure-stabilizing or regulatory role (3). In this regard, phosphorylation of LCb and not of LCa, or the clathrin heavy chain by coated vesicle-associated casein kinase II may be of particular functional significance (4).

The phosphorylation of LCb has been reported in both in vitro and in vivo systems (5–8) and is known to occur on serine residues (6). Our previous analysis of the primary

* This research was supported by grants from the American Cancer Society (to K. D. and P. F.), the National Science Foundation (to F. M. B.), and the National Cancer Institute, National Institutes of Health (to B. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The abbreviations used are: LCa, clathrin light chain a; LCb, clathrin light chain b; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

structures of light chains showed that at positions 9–13 LCb has the sequence Ser-Ser-Ser-Glu-Ser whereas the corresponding region of LCa has the sequence Gly-Gly-Pro-Ala-Leu (9). This amino-terminal region provided a possible site for LCb-specific phosphorylation. Here we present experiments demonstrating that this hypothesis is correct.

EXPERIMENTAL PROCEDURES

Phosphorylation and Purification of Clathrin Light Chains—Clathrin-coated vesicles from three bovine brains were purified and phosphorylated as previously described (5) with the following modifications of the phosphorylation reaction: 2 mg/ml clathrin-coated vesicles, 100 μM [γ-32P]ATP (100 mCi/mmol), 50 μg/ml polylysine (Mw = 40,000). Light chains were isolated from Tris extracts of the coated vesicles by boiling, dialysis against water, and high speed centrifugation as previously described (10). The light chain-containing supernatant was concentrated by ultrafiltration. The light chains were separated from low molecular weight contaminants by HPLC on a reverse phase C3 column (Beckman Instruments), eluting with a gradient of acetonitrile in 0.1% trifluoroacetic acid. Aliquots of 32P-containing HPLC fractions were analyzed by SDS-PAGE followed by autoradiography.

Proteolysis of Phosphorylated LCb—HPLC fractions containing specifically phosphorylated LCb were pooled and dialyzed against water to prepare for cleavage with clostripain or chymotrypsin using a 1:50 (w/w) ratio of enzyme to light chain. Conditions for digestion with clostripain were 0.1 M Tris-HCl, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂, and 1 mM dithiothreitol. After 12 h at 37 °C, clostripain was inactivated by adding iodoacetamide to 1 mM. Conditions for digestion with chymotrypsin were 0.1 M NH₄HCO₃, pH 8.1, for 4.5 h at 37 °C. Chymotrypsin was inactivated by adding trifluoroacetic acid to 0.1%. After inactivation of protease, samples were acidified with trifluoroacetic acid to 0.1%, centrifuged for 10 min in an Eppendorf microcentrifuge, and applied to the reverse phase columns.

Amino Acid Sequence Determination and Identification of Phosphorylated Residues—Amino acid sequences of chymotryptic peptides were determined using a pulsed-liquid sequenator. Radioactivity in each sequencing cycle was quantitated by liquid scintillation counting.

Inhibition of Phosphorylation by Monoclonal Antibodies—Coated vesicles (30 μg) and monoclonal antibodies (11) at varying concentrations were preincubated in phosphorylation buffer (5) for 1 h at 4 °C. The phosphorylation reaction (50 μl total volume) was initiated by adding polylysine to 50 μg/ml and [γ-32P]ATP to 20 μM (100 mCi/mmol), and the reaction continued for 5 min at 30 °C. The reaction was stopped by the addition of 50 μl of 2 X SDS-PAGE sample buffer and boiling for 3 min. 10 μl was analyzed by SDS-PAGE (12). Gels were stained, dried, and autoradiographed for 16–20 h.

RESULTS AND DISCUSSION

Incubation of purified coated vesicles with [γ-32P]ATP resulted in specific phosphorylation of LCb and not of LCa, or the clathrin heavy chain (5). After purification from the coated vesicles, 32P-labeled LCb was first digested with chymotrypsin and the peptides analyzed by reverse phase HPLC. A single peak of radioactivity was detected which eluted with ~35% acetonitrile (Fig. 1a). Sequence analysis of the 32P-containing fractions indicated the presence of at least two peptides. Inspection of the mixed sequence suggested that one peptide corresponded to a region around residues 9–13 and that another corresponded to the sequence around residue 197. Previously we have shown that these two sequences, both of which contain serine, are found in different clostripain fragments of LCb (9). Thus, by first isolating 32P-labeled clostripain fragments and subsequently digesting them with...
Chymotryptic digestion of each of the 32P-labeled chymotryptic peptides showed peaks resulting from heterogeneous cleavage of LCb by pro-terminus (Fig. 2). The major chymotryptic cleavage products of B and C (Fig. 1b). Fractions corresponding to each of these peaks were digested with chymotrypsin and analyzed separately. Chymotryptic digestion of each of A, B, and C produced one major 32P-containing peak (Fig. 1c). Sequencing of each of the 32P-labeled chymotryptic fragments showed they all derived from the amino-terminal region of LCb and that the multiple cleavage products of B and C are conserved between species (9, 13). When compared with other known target sequences of casein kinase II, the phosphorylation site of LCb is similar, having the requisite acidic residues carboxyl terminal to the phosphorylation site (14) (Fig. 2). As previously reported for casein kinase II, heparin and calcium inhibited the phosphorylation of LCb, while polylysine stimulated LCb phosphorylation (15) (not shown).

Recently, Kohtz et al. (15) reported two monoclonal antibodies, specific for LCb, whose binding to coated vesicles inhibited the phosphorylation of LCb. We found that 4 of 14 monoclonal antibodies to LCb inhibit the phosphorylation of LCb. Two of these antibodies are specific for LCb (LCB.3 and X44), and two bind both LCb and LCa (LCB.1 and X43). LCB.1 and LCB.3 were the most potent, showing inhibitory activity at a concentration of 100 μg/ml (Fig. 3b). X43 and X44 became inhibitory at 200 μg/ml (Fig. 3c). In each case, the inhibition is dose-dependent and specific; the phosphorylation of pp50 and tubulin, the other major phosphorylated components of the coated vesicle preparation, is not inhibited. Previously, the LCB.3 and X43 epitopes were mapped to epitopes present on two different carboxyl-terminal fragments of LCb, while the locations of the LCB.1 and X44 epitopes were undetermined (11). In the present study, none of these antibodies bound to a synthetic peptide encompassing the phosphorylation site of LCb, and corresponding to residues 21 (not shown). Therefore, these antibodies are unlikely to compete with casein kinase II at the phosphorylation site. These data are consistent with either of two explanations. Casein kinase II may interact with an extensive region of LCb, carboxyl-terminal to the phosphorylation site, such that binding of antibodies within these regions interferes with phosphorylation. Alternatively, carboxyl-terminal regions of LCb may be sufficiently close to the phosphorylation site that
antibody binding stERICally hinders the binding of the kinase to the phosphorylation site.

Both X43 and X44 have been mapped to epitopes that are cryptic in assembled coated vesicles (11), results which are consistent with a lower affinity of these antibodies for assembled, as compared to free, LCb. In the present study, this affinity limitation is lessened, since the antibody concentration is constant during the course of the phosphorylation reaction. Furthermore, breathing of the clathrin structure under these conditions could allow binding to epitopes that are only transiently accessible.

Immunoelectron microscopy studies have shown that clathrin light chains extend along the length of the proximal arm of the triskelion with the carboxyl terminus of the light chain oriented toward the triskelion vertex (16, 17). This orientation places the amino-terminal phosphorylated serines of LCb near the triskelion elbow, a location where they may influence regional interactions between clathrin heavy and light chains (18). Serines 11 and 13 are only 9 and 7 amino acid residues amino-terminal to a sequence (residues 20–41) that is conserved absolutely between LCa and LCb, and between light chains of rat and bovine origin (9, 13). Phosphorylation of serines 11 and 13 provides a potential mechanism to modify differentially the functions of this region of the light chains.

REFERENCES
1. Pearse, B. M. F., and Crowther, R. A. (1987) Annu. Rev. Biophys. Biophys. Chem. 16, 49–68
2. Ungewickell, E., and Branton, D. (1981) Nature 289, 420–422
3. Kirchhausen, T., and Harrison, S. C. (1981) Cell 23, 755–761
4. Bar-Zvi, D. (1987) Nature 326, 133–134
5. Bar-Zvi, D., and Branton, D. (1986) J. Biol. Chem. 261, 9614–9621
6. Usami, M., Takahashi, A., Kadota, T., and Kadota, K. (1985) J. Biochem. (Tokyo) 97, 1819–1822
7. Schook, W. J., and Puszkin, S. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 8039–8043
8. Cantournet, B., Creuzet, C., Komano, O., and Loeb, J. (1987) FEBS Lett. 220, 143–148
9. Jackson, A. P., Seow, H.-F., Holmes, N. J., Drickamer, K., and Parham, P. (1987) J. Cell Biol. 106, 154–159
10. Brodsky, F. M., Holmes, N. J., and Parham, P. (1983) J. Cell Biol. 96, 911–914
11. Brodsky, F. M., Galloway, C. J., Blank, G. S., Jackson, A. P., Seow, H.-F., Drickamer, K., and Parham, P. (1987) Nature 326, 203–205
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Kirchhausen, T., Scarmato, P., Harrison, S. C., Monroe, J. J., Chow, E. P., Mattaliano, R. J., Ramachandran, K. L., Smart, J. E., Ahn, A. H., and Brodiss, J. (1987) Science 236, 320–324
14. Kuenzel, E. A., Mulligan, J. A., Sommercorn, J., and Krebs, E. G. (1987) J. Biol. Chem. 262, 9136–9140
15. Kohn, D. S., Georgieva-Hanson, V., Kohlz, K. D., Schook, W. J., and Puszkin, S. J. (1987) J. Cell Biol. 104, 897–903
16. Kirchhausen, T., Harrison, S. C., Parham, P., and Brodsky, F. M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 2481–2485
17. Ungewickell, E. (1985) EMBO J. 2, 1401–1408
18. Blank, G. S., and Brodsky, F. M. (1987) J. Cell Biol. 105, 2011–2019
19. Carmichael, D. F., Gaehlen, R. L., Allen, S. M., and Krebs, E. G. (1982) J. Biol. Chem. 257, 10440–10445
20. Hemnings, B. A., Atkin, A., Cohen, P., Rymond, M., and Hofmann, F. (1982) Eur. J. Biochem. 227, 473–481
21. Miggia, F., Donella-Deana, A., and Pinna, L. A. (1978) FEBS Lett. 91, 216–221