Selective Externalization of an ATP-binding Protein Structurally Related to the Clathrin-uncoating ATPase/Heat Shock Protein in Vesicles containing Terminal Transferrin Receptors during Reticulocyte Maturation*

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Transferrin receptors are lost from reticulocytes in vesicles that are released during the final stage of erythocyte maturation (Pan, B. T., and Johnstone, R. M. (1983) Cell 33, 967–977). Transferrin receptor-containing vesicles have a major protein component present in a 1:1 ratio with the receptor that migrates on sodium dodecyl sulfate gels as two polypeptides of Mr = 71,000 and 72,000. The Mr = 71,000/72,000 doublet is indistinguishable from the clathrin-uncoating ATPase/heat shock protein based on cross-reaction with affinity-purified antibody against the uncoating protein, by comparison of peptide maps of the Mr = 72,000 and 71,000 polypeptides and the uncoating protein, and by selective binding of these polypeptides to ATP-agarose. This finding suggests a possible activity of proteins related to the uncoating/heat shock protein family in the disposal of aged membrane proteins by a pathway independent of lysosomes.

Erythrocytes incorporate about $1 \times 10^8$ iron molecules/cell into hemoglobin and transport this iron into the cell utilizing the transferrin receptor and the process of receptor-mediated endocytosis (Jandl and Katz, 1963; Hemmaplardh and Morgan, 1977). Transferrin receptors are present in reticulocytes or erythrocyte precursors at high levels during hemoglobin synthesis and then are dramatically reduced in number during the final stage of erythocyte maturation (Vann Bockmane and Morgan, 1979; Frazier et al., 1982; Enns et al., 1981; Pan and Johnstone, 1983). The transferrin receptors are lost from reticulocytes in the form of vesicles that are released with apparently intact, externally oriented receptor as a major protein component (Pan and Johnstone, 1983; Harding et al., 1983). Vesicles are shed by an unusual pathway that involves fusion of endocytic vesicles to form larger vesicles followed by a second membrane fusion event resulting in budding of externally oriented vesicles to form multivesicular elements (Pan et al., 1985; Harding et al., 1984). Vesicles within multivesicular elements are then shed into the medium when the multivesicular elements fuse with the plasma membrane.

The loss of transferrin receptors from reticulocytes by elimination of receptor-containing vesicles provides an example of removal of a membrane protein by a mechanism independent of degradation within lysosomes. This process may represent a highly specialized adaptation of the erythrocye, but it is more likely that at least some features are generally applied in removal of senescent transferrin receptors and membrane proteins from cells other than erythrocytes. Details are not known of how transferrin receptors are segregated into vesicles or of the signals that target receptors for ejection as opposed to normal recycling. An important clue to these questions may be the presence in receptor-containing vesicles of a cytotoxic polypeptide(s) of Mr = 71,000/72,000 that is approximately in a 1:1 ratio with the receptor polypeptide (Pan and Johnstone, 1983, 1984). The Mr = 71,000/72,000 polypeptides are distinct from the receptor with different peptide maps and no cross-reactivity with antibody against the receptor (Pan and Johnstone, 1984; Johnstone et al., 1984a, 1984b). The Mr = 71,000/72,000 polypeptides are contained within the same vesicles as the transferrin receptor since these polypeptides are retained in vesicles isolated by immunoadsorption with antibody against the transferrin receptor (Pan and Johnstone, 1983). The Mr = 71,000/72,000 polypeptides are localized within these vesicles since these polypeptides are inaccessible to lactoperoxidase-catalyzed radiodiodination of reticulocytes (Pan and Johnstone, 1983), or of isolated vesicles.

A protein of Mr = 72,000 has recently been described as a significant component of erythrocye cytosal and was identified as a clathrin-uncoating ATPase (Davis and Bennett, 1985). The uncoating ATPase is an ATP-binding protein initially described in brain that disassembles clathrin cages and is believed to be involved in recycling of clathrin triskelions from endocytic vesicles back to the plasma membrane (Braell et al., 1984; Schlossman et al., 1984; Schmidt and Rothman, 1985a, 1985b). The clathrin-uncoating protein has recently been identified as a member of a highly conserved family of M, about 70,000 ATP-binding proteins termed heat shock or stress proteins (Ungewickell, 1985; Chappell et al., 1986) which have closely related forms in Escherichia coli, yeast, Drosophila, and vertebrates (reviewed by Craig, 1985). This family includes members that are expressed under normal conditions as well as proteins that are synthesized following a variety of stresses such as heat, incubation with abnormal amino acids, and heavy metal poisoning.

In this report evidence is presented that the clathrin-uncoating ATPase/heat shock protein is structurally related to the protein that is segregated in vesicles containing the terminal transferrin receptors. This finding suggests a possible activity of the protein in the uncoating/heat shock protein family in the disposal of aged or dispensable membrane proteins of reticulocytes and possibly other cells by a pathway independent of lysosomes.

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1 R. M. Johnstone, unpublished data.
EXPERIMENTAL PROCEDURES

Materials—Culture medium was from GIBCO. Sheep transferrin and anti-transferrin receptor antibody were prepared, respectively, as described (Pan et al., 1983; Johnstone et al., 1984). Human erythrocyte clathrin-uncoating ATPase was purified as described (Davis and Bennett, 1985). Na\(^{125}\)I was from Amersham Corp. Hydroxylapatite (high resolution) was from Behring Diagnostics. Plastic thin-layer sheets coated with 0.1 mm of cellulose was from E. Merck. Diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, dithiothreitol, and ATP-agarose (linked through C-8) were from Sigma. Cyanogen bromide-activated Sepharose 4B, Dextran 500, Nitrocellulose paper, electrophoresis reagents, and silver-staining reagents were from Bio-Rad. Sucrose and ammonium sulfate were from Schwarz/Mann. \(\alpha\)-Chymotrypsin (44 units/mg) was from Worthington. Cynogen bromide-activated Sepharose 4B, Dextran 500, Protein A, Protein A-Sepharose, Percoll, and Sephacyrl S-400 were from Pharmacia P-L Biochemicals. DEAE-53-cellulose was from Whatman. Erythrocyte ghosts were prepared as described (Bennett, 1987).

Methods—To isolate transferrin receptor vesicles from sheep reticulocytes, reticulocyte production was induced by repeated phlebotomy, and the reticulocytes were isolated by differential centrifugation (Pan et al., 1983; Benderoff et al., 1978). Vesicles were released from reticulocytes by incubation for 18 h at 37 °C as described (Pan et al., 1983). The reticulocytes were removed by two centrifugation steps at 12,000 \(\times\) g for 5 min, and the externalized vesicles were collected by centrifugation for 90 min at 100,000 \(\times\) g. SDS-polycrylamide electrophoresis was performed on 3.5–17% gradient slab gels with the buffers of Fairbanks et al. (1971). Immunoblot analysis was performed by electrophoretically transferring proteins from SDS gels to nitrocellulose paper (Towbin et al., 1979) using 1 \(\mu\)g/ml IgG and \(^{125}\)I-labeled Protein A as described (Bennett and Davis, 1981). Polypeptides on nitrocellulose were stained with Ponceau S (0.2% (w/v) acetic acid). Protein was determined by the methods of Bradford (1976), Lowry et al. (1951), or absorbance at 280 nm. The concentration of purified uncoating protein was determined by the method of Bradford (Schlossman et al., 1984) and scans of Coomassie Blue-stained gels were used to determine the purity. Rabbit antiserum against human erythrocyte uncoating ATPase was prepared by immunization with uncoater/ATPase polypeptide that was cut out from preparative SDS-polyacrylamide gels and injected as described (Bennett and Stenbuck, 1979). Affinity-purification antibody against the uncoater/ATPase was isolated by affinity chromatography with human erythrocyte uncoater/ATPase coupled to Sepharose 4B as described (Bennett and Stenbuck, 1979) except that the antibody was eluted with 4 M MgCl\(_2\) instead of 1 M acetic acid.

RESULTS

A Protein Structurally Related to the Uncoating/Heat Shock Protein Is a Component of the Transferrin Receptor-containing Vesicles—Vesicles shed from reticulocytes during incubation in vitro at 37 °C contain two major bands in approximately a 1:1 ratio; one of \(M_r\) \(\sim 93,000\) (the transferrin receptor) and another that migrates as a doublet of \(M_r\) \(\sim 71,000\) and 72,000 (Fig. 1, lane 7) (Pan and Johnstone, 1984). These bands are segregated in the vesicles with a remarkable selectivity; the major cytosolic and membrane polypeptides such as hemoglobin, catalase, and spectrin which are present in intact erythrocytes in considerable excess over the two vesicle bands are largely excluded from vesicles (Fig. 1) (Pan and Johnstone, 1984).

The \(M_r\) is 71,000/72,000 doublet has been identified as a member of the uncoating ATPase/heat shock protein family based on cross-reaction with affinity-purified antibody against the uncoating protein (Figs. 1 and 2), by comparison of peptide maps of the \(M_r\) \(\sim 72,000\) and 71,000 polypeptides and the uncoating protein (Fig. 3), and by ATP affinity chromatography (Fig. 4). The \(M_r\) \(\sim 72,000\) component of the 71,000/72,000 doublet cross-reacts with affinity-purified antibodies raised against the erythrocyte-uncoating ATPase (Fig. 1). The explanation for the lack of reaction with the
M = 71,000 generated from homogeneous uncoating protein by cleavage with chymotrypsin (Fig. 2). Furthermore, it is likely that the $M_1 = 71,000$ vesicle polypeptide that does not cross-react with antibody is a proteolytic fragment of the higher molecular weight cross-reacting band since peptide maps of these polypeptides were very similar or identical, with the exception of two spots that are present only in the $M_1 = 72,000$ polypeptide (Fig. 3). Degradation of the purified uncoater/heat shock protein occurs during storage and may result from an endogenous protease activity of this protein (Mitchell et al., 1985).

Peptide maps of uncoating protein are closely related to those of both components of the vesicle 71,000/72,000 doublet (Fig. 3). All of the spots derived from the uncoating protein appear in maps of the vesicle polypeptides, although the relative intensities of labeling vary in some cases. The basis for variation in iodination is not clear, but could reflect genuine differences in structure. The uncoater/heat shock protein family includes both "cognate" or constitutively expressed forms as well as a closely related isoform that is induced with stress and has a slightly faster mobility on SDS gels. The cognate and stress-induced polypeptides share ability to bind to clathrin (Ungewickell, 1985) but have distinct one-dimensional peptide maps. The difference observed in peptide maps in this study is considerably less, and it is likely that both the uncoater and the vesicle polypeptides represent the constitutively expressed member(s) of this protein family. The uncoater/heat shock protein also has isoelectric variants (Hightower and White, 1981; Ungewickell, 1985), and it is possible that the differences in peptide maps reflect the same diversity that causes variation in isoelectric point.

The vesicle $M_1 = 71,000/72,000$ polypeptides also share with the clathrin uncoater/ATPase the capacity to bind ATP. These polypeptides bind selectively to ATP-affinity columns and are eluted with ATP (Fig. 4). The transferrin receptor, in contrast, was not adsorbed to the ATP column (Fig. 4).

**Attempts to Demonstrate a Complex of $M_1 = 71,000/72,000$ Polypeptides with the Transferrin Receptor**—The cosegregation of transferrin receptor with the $M_1 = 71,000/72,000$ polypeptides implies that at some stage these proteins may have been associated with each other. The possibility that such a complex was preserved in the vesicles was examined by immunoprecipitating 51I-labeled vesicles solubilized in Triton X-100 with antibody either against the receptor or the uncoating protein. Both antibodies were capable of immunoprecipitating a major portion of their antigens but did not precipitate other proteins. Similar results were obtained in both laboratories. Thus, the $M_1 = 71,000/72,000$ polypeptides and receptor are not linked in a stable complex that survives vesicle storage and the rigors of detergent solubilization and immunoprecipitation.

**DISCUSSION**

Transferrin receptors in reticulocytes represent an experiment of nature where a cohort of membrane receptors has been kinetically isolated with no renewal by protein synthesis and these receptors are lost synchronously during the final stages of erythrocyte maturation. The fates of these terminal or senescent receptors involves diversion of receptor-containing endocytic vesicles to large multivesicular elements instead of their usual circuit back to the plasma membrane (Pan et al., 1985; Harding et al., 1984). The multivesicular bodies then fuse with the plasma membrane, and vesicles are released into the medium. The observation that proteins structurally re-

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$^3$J. Q. Davis, D. Dansereau, R. M. Johnstone, and V. Bennett, unpublished data.
$M_r = 72,000$ Heat Shock Protein and the Terminal Trans ferrin Receptor

FIG. 5. Possible scheme for the externalization of the transc ferrin receptor in vesicles. The erythrocyte protein related to the clathrin-uncoating protein (UC) first uncoats endocytosed transferrin receptor (R) and transferrin (TF) containing coated vesicles (clathrin coat represented by a bold line) then fuse to form larger vesicles. UC then tags aged or senescent receptors, targeting them for a re endovesiculation event forming multivesicular elements. The multivesicular structures then fuse with the plasma membrane with subsequent secretion of the small vesicles containing externally oriented receptor and internal uncoating protein.

...related to the clathrin-uncoating/heat shock protein are cosegregated with the receptor in a 1:1 ratio in the everted vesicles and that these are the major proteins in these vesicles suggests that these proteins play some role in directing receptor traffic.

It is conceivable that the $M_r = 71,000/72,000$ polypeptides are associated with the receptor during conventional recycling and plays no specific role in targeting senescent receptors for ejection. In this case these proteins may have a function in the normal receptor cycle, such as lateral association of receptors in the plasma membrane or recycling endocytotic vesicles back to the plasma membrane.

...Another, more likely, possibility is that the $M_r = 71,000/72,000$ polypeptides are directly involved in elimination of terminal transferrin receptors and do not interact with receptors during their functional life. Such a role in detection and removal of an aged protein is consistent with the observation that synthesis of proteins related to the uncoater/heat shock protein is activated by microinjection of denatured proteins, and with the fact that the diverse perturbations that induce stress-related proteins all have in common alteration of protein structure (Ananthan et al., 1986). The erythrocyte abortly loses its protein synthesis machinery as well as its lysosomes in the final stages of differentiation and would be expected to have a special need for dealing with populations of proteins that subsequently enter senescence at nearly the same time. The large number of copies of uncoater/heat shock protein expressed in these cells during differentiation (Singh and Yu, 1984; Morimoto and Fodor, 1984) could be explained if this protein is involved in clearing the cell of terminal proteins. The mature human erythrocyte, for example, has 250,000 copies of uncoater/heat shock protein and only 6,000 copies of clathrin triskelions, suggesting some function for the uncoater/heat shock protein that is not limited to disassembly of clathrin cages.

It will be important in the future to determine if and how the $M_r = 71,000/72,000$ polypeptides select aged receptors and direct them into the multivesicular compartment. One possible working hypothesis (Fig. 5) is that the $M_r = 71,000/72,000$ polypeptides, in the process of disassembling clathrin-coated vesicles, recognizes and binds to aged receptors and then promotes fusion of these vesicles with other vesicles containing an uncoater/heat shock protein-receptor complex. Eventually such a process would lead to large membrane vesicles that could endovesiculate to form multivesicular bodies by the same mechanism of fusion of the cytoplasmic membrane surfaces mediated by $M_r = 71,000/72,000$ polypeptides.

The uncoater/heat shock protein interacts with membrane proteins in nucleated cells. A cell surface glycoprotein of mouse 3T3 cells becomes associated with the constitutively expressed $M_r = 72,000$ and heat-induced $M_r = 70,000$ polypeptides following heat treatment but not in unstressed cells (Hughes and August, 1982). This membrane protein may be distinct from the transferrin receptor, although it is concentrated in coated pits on the cell surface (Murphy et al., 1983). This observation indicates that the mechanism for removal of transferrin receptors from reticulocytes reflects a general pathway for clearing of at least certain membrane proteins from the cell. It will be of interest to determine if aged or denatured membrane proteins are also ejected from the cell in vesicles as is the case in erythrocyte or if these abnormal proteins are degraded by lysosomes.

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