Exclusion of Erythrocyte-specific Membrane Proteins from Clathrin-coated Pits during Differentiation of Human Erythroleukemic Cells

LINDA M. MARSHALL, ÅSA THURESON-KLEIN, and RICHARD C. HUNT
Departments of Biochemistry and Pharmacology, The University of Mississippi Medical Center, Jackson, Mississippi 39216. Dr. Marshall's present address is Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284.

ABSTRACT When human erythroleukemic cells are induced to differentiate, they produce globin and redistribute glycophorin and spectrin to one pole of the cell. This process was accompanied by an alteration in the clathrin-coated pits at the cell surface. In nondifferentiating cells, receptors for Concanavalin A have been shown, using electron microscopy, to be concentrated into coated pits and rapidly internalized. Glycophorin was also internalized via coated pits, but was not greatly concentrated into these portions of the surface membrane. Ligands attached to glycophorin were, therefore, cleared from the cell surface more slowly than Concanavalin A. In nondifferentiating cells, immunoelectron microscopy showed that spectrin is largely excluded from coated pits. After erythroid differentiation proceeded for several days, glycophorin was totally excluded from the coated pits along with spectrin. This did not reflect a general cessation of endocytosis, however, because Concanavalin A receptors continued to be internalized. It is possible that the specific exclusion of glycophorin from coated pits is part of the remodeling process that occurs when the precursor cell membrane differentiates into that of the mature erythrocyte.

The later stages of the differentiation process that culminates in the formation of the fully mature erythrocyte are characterized by a major remodeling of the cell's plasma membrane. Prior to the formation of the reticulocyte by enucleation of the precursor cell, those proteins that are characteristic of the membrane of the mature erythrocyte (such as glycophorin and spectrin) are sequestered to that part of the membrane that will surround the reticulocyte and away from the membrane that surrounds the nucleus (11, 22, 23).

The membrane of the fully mature erythrocyte is unusual in the simplicity of its protein composition when compared with plasma membranes of nucleated cells (15) and it is likely that the final stages of differentiation of the membrane are also accompanied by the loss of many proteins, the functions of which are no longer required. Thus, for example, the transferrin receptor, which is abundant in the membrane of the reticulocyte, is absent from the membrane of the fully mature erythrocyte (7, 16). These processes, the conservation of some proteins on the cell surface and their sequestration to one portion of the membrane at the same time as other proteins are being removed are carried out by unknown mechanisms, but may involve membrane endocytotic structures visualized ultrastructurally as clathrin-coated pits. For example, if clathrin-coated pits were to generate a flow of membrane components toward one site on the cell surface, it is possible that membrane proteins would be swept along in this flow and aggregated at one pole of the cell (4, 22). In addition, coated pits may be involved in the conservation of certain proteins at the cell surface concomitant with the removal of others. This could occur by the restraining of some proteins from entering the coated pits whereas others are actively concentrated into these structures.

Some aspects of erythroid differentiation in vivo are mimicked by the cultured K562 human erythroleukemic cell line. These cells manufacture spectrin (12), glycophorin (9), and a surface glycoprotein designated gp105 (3, 25) that bears similar carbohydrate chains to those of band 3 protein (8), although band 3 protein itself does not occur in these cells (25). After induction of erythroid differentiation by growth of the cells in hemin- (18) or sodium butyrate-containing medium (2), some of the cells redistribute globin, spectrin, and glycophorin into a reticulocyte-like cell that subsequently buds from the parent cell (12).

In this report, we show that as K562 cells differentiate, the

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 JUNE 1984 2055-2063

endocytosis of glycophorin, a protein characteristic of the mature erythrocyte membrane, is curtailed by excluding it from areas of the membrane that are lined with clathrin. As a result, glycophorin becomes distributed over areas of the surface membrane that are lined by spectrin, the major component of the submembranous cytoskeleton (15). In contrast, the endocytosis of another membrane protein is maintained, Concanavalin A receptors being actively concentrated into coated pits throughout the period of differentiation that we have investigated.

MATERIALS AND METHODS

Cell Culture: K562 cells were kindly donated by Dr. T. Rutherford (Radcliffe Infirmary, Oxford, England) and grown in Corning tissue culture flasks containing supplemented RPMI 1640 medium (Gibco Laboratories, Grand Island, NY). For induction of differentiation, cells were grown in medium containing 0.05 mM hemin (Sigma Chemical Co., St. Louis, MO) (18).

Production of Antibodies: Purified human erythrocyte membranes (5), or spectrin purified by the method of Marchesi (14), were suspended in phosphate buffered saline (PBS) at 1 mg/ml, and then emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected intramuscularly into male New Zealand white rabbits at 3-wk intervals. Sera were purified and characterized by standard techniques (10). Antispectrin antibody was purified further by affinity chromatography on a spectrin-Sepharose column.

Characterization of the antisera. One antiserum used in these studies was raised by injecting washed human erythrocyte membranes into rabbits and was characterized by immune precipitation of lactoperoxidase-iodinated cells. K562 cells were labeled on their external surfaces using $Na^{125}I$, lactoperoxidase, and glucose oxidase (12), and were dissolved and incubated with the antiserum as previously described (12). Only two labeled proteins were immune precipitated and these co-migrated on SDS-polyacrylamide gels with PAS-1 and PAS-2 of the erythrocyte membrane (Fig. 1). Further confirmation of the specificity of this antiserum, as far as K562 cell external surface molecules are concerned, was obtained by absorbing the antiserum with glycophorin purified from erythrocyte membranes according to the method of Segrest and colleagues (21). The preparation of glycophorin used contained only two components, which co-migrated on SDS-polyacrylamide gels with PAS-1 and PAS-2. No contaminating band 3 was detected (data not shown). Preabsorption of the antibody with glycophorin abolished the characteristic surface fluorescence observed when K562 cells were incubated with the primary antibody and fluorescein-conjugated goat anti-rabbit IgG, suggesting that the primary antibody was glycophorin-specific (Fig. 2). One of the antisera used in these studies, therefore, detects only PAS-1 and PAS-2 (the dimer and monomer of glycophorin) on the surface of K562 cells. It is referred to in this paper as the glycophorin-detecting antiserum to emphasize that, although it detects only glycophorin on the surface of K562 cells, it does bind to other antigens in erythrocytes.

Our second antiserum was raised in rabbits by injecting purified human erythrocyte spectrin. The IgG fraction was prepared (10) and antispectrin antibodies isolated by affinity chromatography on a spectrin-Sepharose column. When this antiserum was incubated with [^{35}S]methionine-labeled K562 cell extracts, the only proteins to be specifically precipitated were the two polypeptides of spectrin (bands 1 and 2) and a third polypeptide intermediate in size between bands 1 and 2, which may be another spectrin polypeptide characteristic of immature erythroid cells. Again, preabsorption with purified antigen, bands 1 and 2 of spectrin, abolished the interaction of the antibody with Triton-permeabilized K562 cells (see reference 12).

Electron Microscopy: Depending upon the particular procedure, 5 × 10^4 cells were fixed either before or after specific labeling treatments, by suspension in 2% glutaraldehyde in PBS for 15 min at 4°C. The cells were pelleted gently using a bench centrifuge, washed in isotonic phosphate-buffered sucrose, postfixed in 2% OsO_4 in cacodylate buffer for 20 min at 23°C, and then resuspended in 0.1 ml of molten 1% noble agar dissolved in water. 1-mm cubes were cut from the agar, dehydrated in graded ethanol followed by equilibration in complete Spurr low viscosity resin at 4°C overnight. Blocks were embedded in fresh Spurr resin, allowed to polymerize for 16 h at 60°C, and thin sections were cut using a diamond knife and an LKB ultramicrotome. Silver sections were usually observed unstained for maximum ferritin contrast since coated areas are readily identified in the absence of staining; when staining was required, uranyl acetate and lead citrate were used. Grids were viewed at 60 Kv with a Zeiss EM 10 electron microscope.

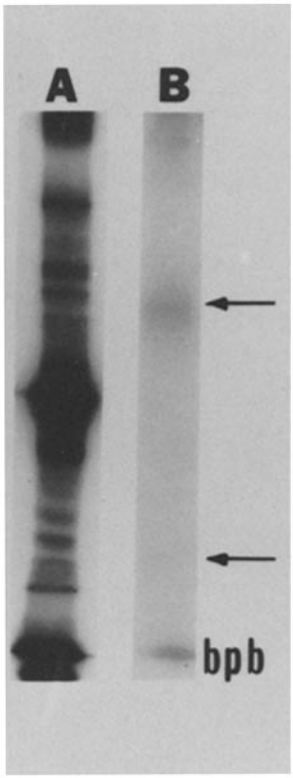

FIGURE 1 Immune precipitation of K562 cell extracts by the glycophorin-detecting antibody. K562 cells were labeled by lactoperoxidase-catalyzed iodination and either analyzed by 7.5% polyacrylamide SDS gel electrophoresis (*a*) or dissolved in NP-40-containing buffer and incubated with the antibody. The washed immune precipitates were then analyzed using the same polyacrylamide gel system (*b*). The upper and lower arrows indicate the positions of PAS-1 and PAS-2, respectively. *bpb* indicates the position of the Bromophenol blue tracking dye.

Immune Electron Microscopy and SDS PAGE: In studies of the distribution of glycophorin on the cell surface, cells were first incubated at 4°C with primary rabbit IgG for 1 h. After washing three times in PBS, the distribution of the primary antibody was detected using either ferritin-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) or colloidal gold-protein A prepared according to the procedure of Roth et al. (17). The cells were then washed and fixed with 2% glutaraldehyde. In some cases the cells were warmed to 37°C for various periods after incubation with both primary and secondary antibody while, in other cases, the cells were fixed prior to incubation with primary antibody. Similar procedures were used to locate Concanavalin A (Con A) receptors except that cells were incubated directly with ferritin-conjugated Con A or succinyl Con A (both obtained from Polysciences, Warrington, PA). Spectrin was detected by fixation of cells in glutaraldehyde followed by incubation at room temperature in PBS containing 0.05% Triton X-100 for 5 min. The permeabilized cells were then incubated with affinity-purified rabbit anti-spectrin IgG, washed in PBS, and then incubated with ferritin-conjugated goat anti-rabbit IgG or colloidal gold-protein A.

Electrophoresis in 7.5% polyacrylamide gels was performed as described by Laemmli (13).

RESULTS

Changes occur in clathrin-coated pits of K562 cells undergoing erythroid differentiation. The distribution of glycophorin molecules on the external surface of K562 erythroleukemic cells was investigated before and at various times after the induction of differentiation, using a glycophorin-detecting antiserum and electron microscopy. K562 cells were grown in either the absence of inducer or induced to differentiate along the erythroid differentiation pathway for 96 h, then cooled on ice, and incubated with a glycophorin-detecting antiserum. The primary antiserum was located using ferritin-conjugated goat anti-rabbit IgG as an electron-dense marker. In nondifferentiating and differentiating cells, the electron-dense probe was found over the cell surface, often in small patches. When the surfaces of the nondifferentiating and differentiating cells were observed at greater magnification, a difference was detected in the distribution of erythrocyte membrane antigens in the two cell types. In the nondifferentiating cells, ferritin

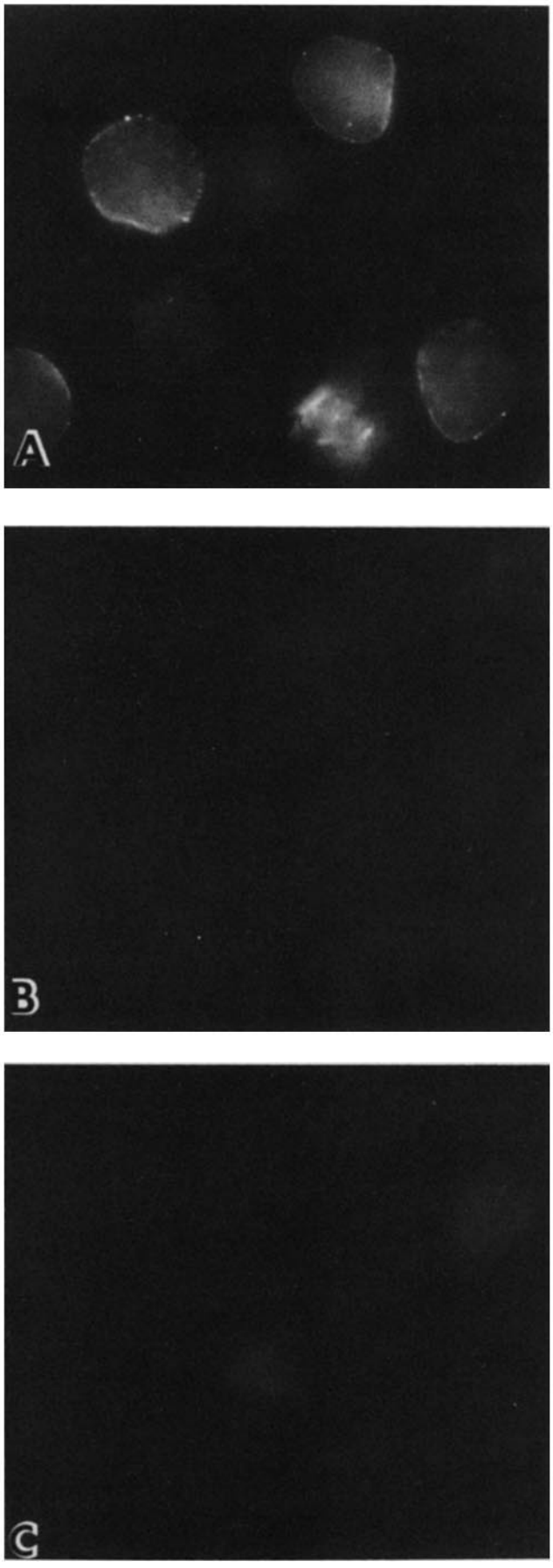

FIGURE 2 Specificity of the glycophorin-detecting antibody. K562 cells were incubated with (a) antibody followed by goat anti-rabbit IgG conjugated to fluorescein; (b) antibody that had been preabsorbed with purified glycophorin (the only components detectable on SDS PAGE being PAS-1 and PAS-2) followed by fluorescein-conjugated goat anti-rabbit IgG; (c) fluorescein-conjugated goat anti-rabbit IgG. All exposures were made for the same length of time. × 1,400.

was observed to be distributed over the surface membrane including over the surface of the coated pits (Fig. 3*a*). The electron-dense particles were observed in deep coated invaginations (Fig. 3*b*) and in coated vesicle profiles (Fig. 3*c*), the latter representing either tangential sections through deep invaginations still connected to the cell surface so that ferritin-IgG can penetrate, or coated vesicles that had been pinched off after labeling despite the low temperature at which the cells were being incubated. In contrast to the distribution of ferritin-IgG seen in nondifferentiating cells, the differentiating cells bound ferritin-IgG along the noncoated areas of membrane but little or no ferritin was bound to the coated areas (Fig. 3, *d–g* and Table I). No electron dense material was observed at the surface in the absence of primary antibody (Fig. 3*h*) nor when the antibody was preabsorbed with purified glycophorin. It, therefore, appears that glycophorin is only associated with clathrin-coated pits in the nondifferentiating cells. These data suggest that the glycophorin may be internalized by the clathrin-coated pits of nondifferentiating cells but not by these structures in K562 cells that are undergoing erythroid differentiation.

Concanavalin A receptors are incorporated into coated areas of both nondifferentiating and differentiating K562 cells. The exclusion of glycophorin from the coated pits of differentiating cells, after differentiation has proceeded for several days, might result from a general inhibition of the movement of membrane proteins into coated areas and their subsequent endocytosis. Alternatively, the lack of these proteins in the coated areas might be a specific phenomenon possibly associated with the reorganization of membrane proteins during erythroid differentiation. To determine the specificity of the change that occurs with differentiation, we have used a more general ligand that attaches to cell surface glycoproteins; this probe is Concanavalin A conjugated to ferritin (fer-Con A). Fig. 4, *a* and *b* show coated areas of nondifferentiating and differentiating cells after incubation with fer-Con A at 4°C. The coated areas are very similar in the two cell types: both contain ferritin grains. This suggests that the exclusion of glycophorin from the coated areas is, indeed, specific to these proteins and that other membrane proteins are still being actively taken up by differentiating cells. It is noteworthy that in both cell types the Con A receptors are located primarily in the coated pits and there is very little ferritin on the uncoated cell surface (Table I). It is possible that this concentration into coated areas could arise from the cross-linking action of Con A and subsequent movement of the cross-linked complexes into the pits despite the cells being incubated at 4°C; this is unlikely, however, since succinyl Con A, which behaves as a monovalent ligand and should, therefore, not cross-link its receptors, reveals an identical distribution of Con A receptors in nondifferentiating and differentiating cells (Fig. 4, *c* and *d*). Furthermore, glutaraldehyde-fixed cells show the same distribution of fer-Con A (see below). Thus, even in the absence of the tetravalent ligand, Con A receptors are concentrated into coated areas. Receptors for low density

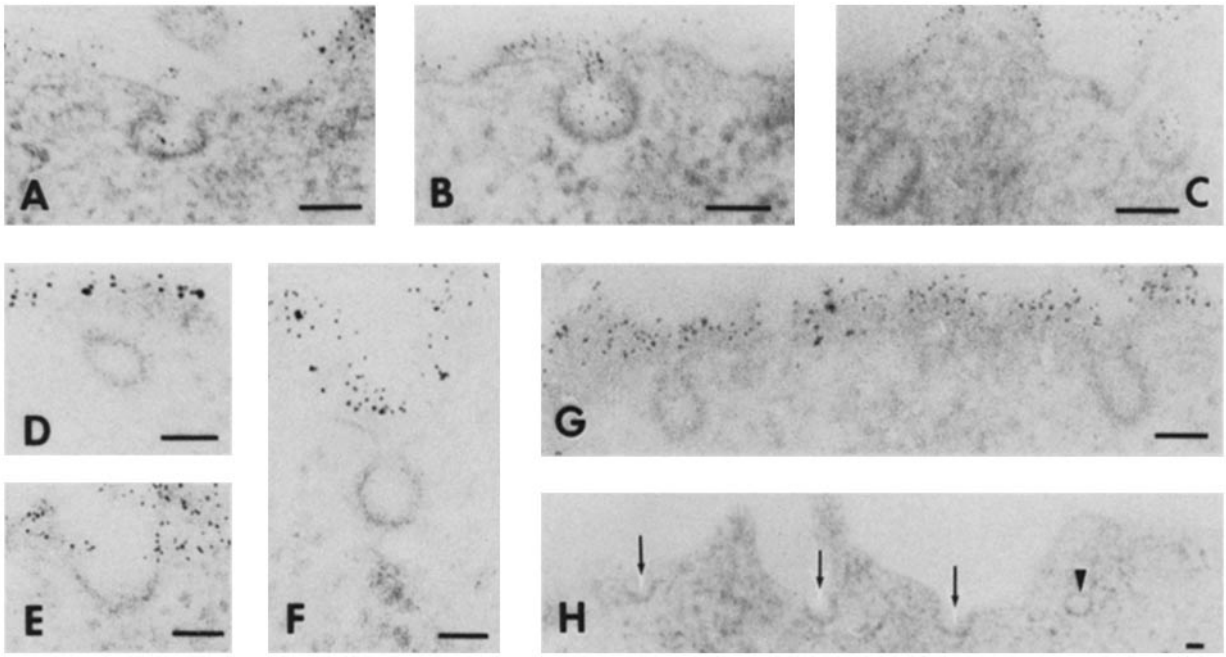

FIGURE 3 Distribution of glycophorins over the surface of the nondifferentiating and differentiating K562 cells. Cells were incubated at 4°C with glycophorin-detecting antibody and subsequently with ferritin-conjugated goat anti-rabbit IgG. The thin sections are unstained. (*A*) The surface of a nondifferentiating cell including a coated pit. Note that ferritin is located in the pit. (*B*) The surface of a nondifferentiating cell including a deep coated pit. As in Fig. 3*A*, the pit is lined with ferritin which also occurs on the cell surface. (*C*) Coated-vesicle profiles containing ferritin in nondifferentiating cells. (*D*) Coated vesicle profile from a K562 cell that had been differentiating for 96 h. No ferritin is in the vesicle though it lines the cell surface. (*E*) An open coated pit from a 96 hour differentiating cell. Again no ferritin is seen in the pit. (*F*) A deep coated pit from a 96 hour differentiating cell. (*G*) Coated pits from 96-h differentiating cells. In both pits shown there is no ferritin associated with the coated area. (*H*) The surface of K562 cells incubated with phosphate buffered saline instead of the primary antibody and then treated as above. Coated pits are designated by arrows and a coated vesicle profile by an arrowhead. Bars, 0.05 μm. × 160,000 (*A–C*); × 150,000 (*D*); × 130,000 (*E* and *F*); × 140,000 (*G*); × 40,000 (*H*).

TABLE I

Distribution of Glycophorin and Con A Receptors on the Surface of Nondifferentiating and Differentiating K562 Cells

	Nondifferentiating		Differentiating	
	Uncoated	Coated	Uncoated	Coated
Glycophorins	6.8 (1,054)	12.6 (176)	6.7 (991)	0.5 (9)
Concanavalin A receptors	2.8 (414)	35.3 (528)	0.8 (116)	87.5 (1,137)

Nondifferentiating and differentiating cells were labeled at 4°C using either glycophorin-detecting antibody and ferritin-conjugated anti-rabbit IgG or fer Con A. After processing for electron microscopy cells were selected at random and regions of the membrane profile photographed in the electron microscope. The lengths of the uncoated and coated membrane profiles were measured and the number of ferritin grains associated with each type of profile counted. The figures are expressed as ferritin grains per micron of membrane profile whereas those in parentheses are the total number of ferritin particles counted. In each case 150 μm of cell surface was quantitated, which contained ~14 μm of coated membrane. The average length of a coated pit profile is 0.125 μm.

lipoprotein have also been shown to be distributed over coated areas in a similar manner (1).

Endocytosis of Glycophorin by K562 Cells

To reveal whether the glycophorin molecules that were found in the clathrin-coated pits of cells labeled at 4°C were subsequently endocytosed, K562 cells were labeled as described above at 4°C using the glycophorin-detecting antiserum, which was itself detected using colloidal gold-conjugated protein A. The cells were then warmed to 37°C for various periods of time, fixed, and processed for electron microscopy. In nondifferentiating cells, soon after warming to 37°C, colloidal gold was located on the cell surface and could be seen, as expected, in coated pits (Fig. 5, *a* and *b*). In Fig. 5*b*, which shows a cell after 20 min at 37°C, a gold particle is seen being delivered from a coated vesicle to a multivesicular structure. Further incubation for a total of 1 h at 37°C resulted in the partial clearing of the gold particles from the cell surface and their movement to regularly shaped multivesicular bodies (Fig. 5*c*). After 1 h at 37°C, gold particles were almost always associated with the periphery of the multivesicular body rather than the central matrix, which suggests that the gold particle remains associated with the antibody-glycophorin complex and was, therefore, still detecting the location of glycophorin. After 6 h at 37°C, the gold particles in the multivesicular bodies were no longer membrane-associated, indicating that the receptor-ligand complex had dissociated (Fig. 6, *a* and *b*). In contrast, the surfaces of differentiating cells remained lined with gold particles even after 6 h at 37°C (Fig. 6*c*), and no internal gold particles were detected.

When cells were incubated with fer-Con A or fer-succinyl Con A at 4°C and the temperature was then raised to 37°C for various periods of time, the situation was somewhat different from that observed with the antiglycophorin IgG.

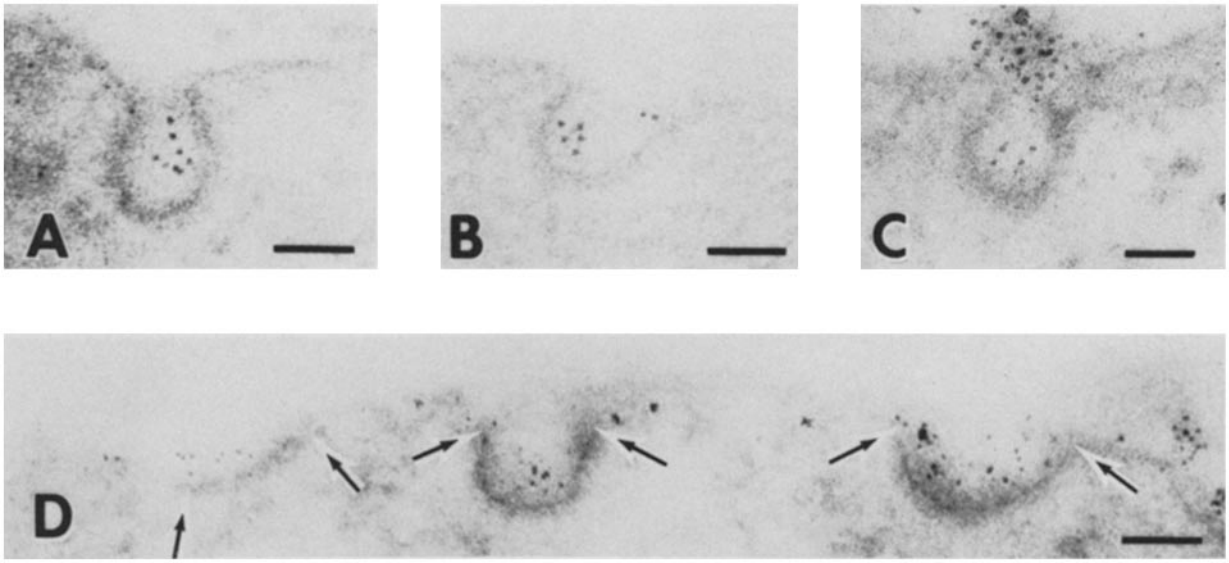

FIGURE 4 Concanavalin A receptors on the surface of nondifferentiating and differentiating K562 cells. Cells were incubated at 4°C with ferritin-conjugated Concanavalin A (fer-Con A) or with ferritin-conjugated succinyl Concanavalin A. (*A*) A coated pit at the surface of a nondifferentiating cell incubated with fer-Con A; (*B*) a coated area from a cell that has been differentiating for 96 h prior to labeling with fer-Con A; (*C*) a coated area from a nondifferentiating cell, incubated with succinyl-fer-Con A; (*D*) Coated areas from a cell differentiating for 96 h and then incubated with succinyl-fer-Con A. Arrows delineate coated areas of the membrane. Bar, 0.05 μm. × 210,000.

Within 5 min of incubation at 37°C all of the labeled Con A was cleared from the cell surface and ferritin associated with Con A or succinyl Con A was seen in the electron-dense lysosomes of both nondifferentiating and differentiating cells (Fig. 6, *d* and *e*).

Distribution of Glycophorin and Concanavalin A Receptors in Glutaraldehyde-fixed K562 Cells

A clear distinction is evident between the incorporation of glycophorin into the coated pits of nondifferentiating K562 cells and its exclusion from coated pits of cells induced to differentiate along the erythroid differentiation pathway when hemin is included in the growth medium. However, even though the cells were maintained at 4°C, there is the possibility that the primary and secondary antibody technique resulted in the incorporation of membrane proteins into coated pits in the nondifferentiating cells as a result of cross-linking by the polyvalent antibody. Furthermore, Con A may induce endocytosis which it is known to do in other cell types (6), although the use of succinyl-Con A in our investigations should eliminate the possibility of the stimulation of new coated pit formation as a result of cross-linking by a polyvalent lectin. To rule out these possibilities further, the distribution of glycophorin and Con A receptors on the surfaces of glutaraldehyde-fixed cells was investigated. In nondifferentiating K562 cells, glycophorin was distributed over the cell surface and in the coated pits (Fig. 7 *a*); however, it appeared to be excluded from the pits of the fixed differentiating cells (Fig. 7, *b–d*), as found with differentiating cells incubated at 4°C. Con A receptors were again found predominantly associated with the coated areas of both cell types (Fig. 7, *e* and *f*).

Distribution of Spectrin Associated with the Membranes of Nondifferentiating and Differentiating K562 Cells

To locate membrane-associated spectrin, nondifferentiating and differentiating K562 cells were fixed in glutaraldehyde and then rendered permeable to antibody by dissolving the lipid bilayer with Triton X-100. The cells were then incubated with specific antispectrin IgG followed by the ferritin second antibody conjugate or protein A-colloidal gold. Spectrin was not found to be associated with the clathrin-coated areas of either nondifferentiating cells (Fig. 8, *a–d*) or differentiating cells (Fig. 8, *e–j*).

This suggests, most probably, that the spectrin-containing cytoskeleton lines the uncoated regions of the plasma membrane but is excluded from areas of membrane specialized for endocytosis, although there is also the possibility that spectrin is present in the coated areas but is not detected by the antibody because of the presence of some component of the coated pit.

DISCUSSION

When an erythroid cell embarks upon the final stages of differentiation, culminating in a terminal unequal cell division to form a reticulocyte, it carries out several major processes as far as the membrane proteins are concerned. First, it sequesters those proteins (glycophorin and spectrin) that are to be constituents of the membrane of the mature erythrocyte into the portion of the plasma membrane that surrounds the incipient reticulocyte (11, 22, 23). Second, proteins that are constituents of the membrane of cells at earlier stages of erythroid differentiation but are not components of the mature erythrocyte membrane are removed. Third, the cell must either cease endocytosis of the membrane proteins that are to remain as constituents of the mature erythrocyte, or if they are endocytosed, they must be recycled intact to the cell surface. Finally, the membrane proteins must undergo a process of concentration such that a submembranous cytoskeleton is formed that lines the inner surface of the entire membrane of the reticulocyte.

Many of these processes may be achieved by selective endocytosis; for example, it has been suggested that selective endocytosis may account for capping of surface receptors in which cross-linked surface antigens move to one pole of the

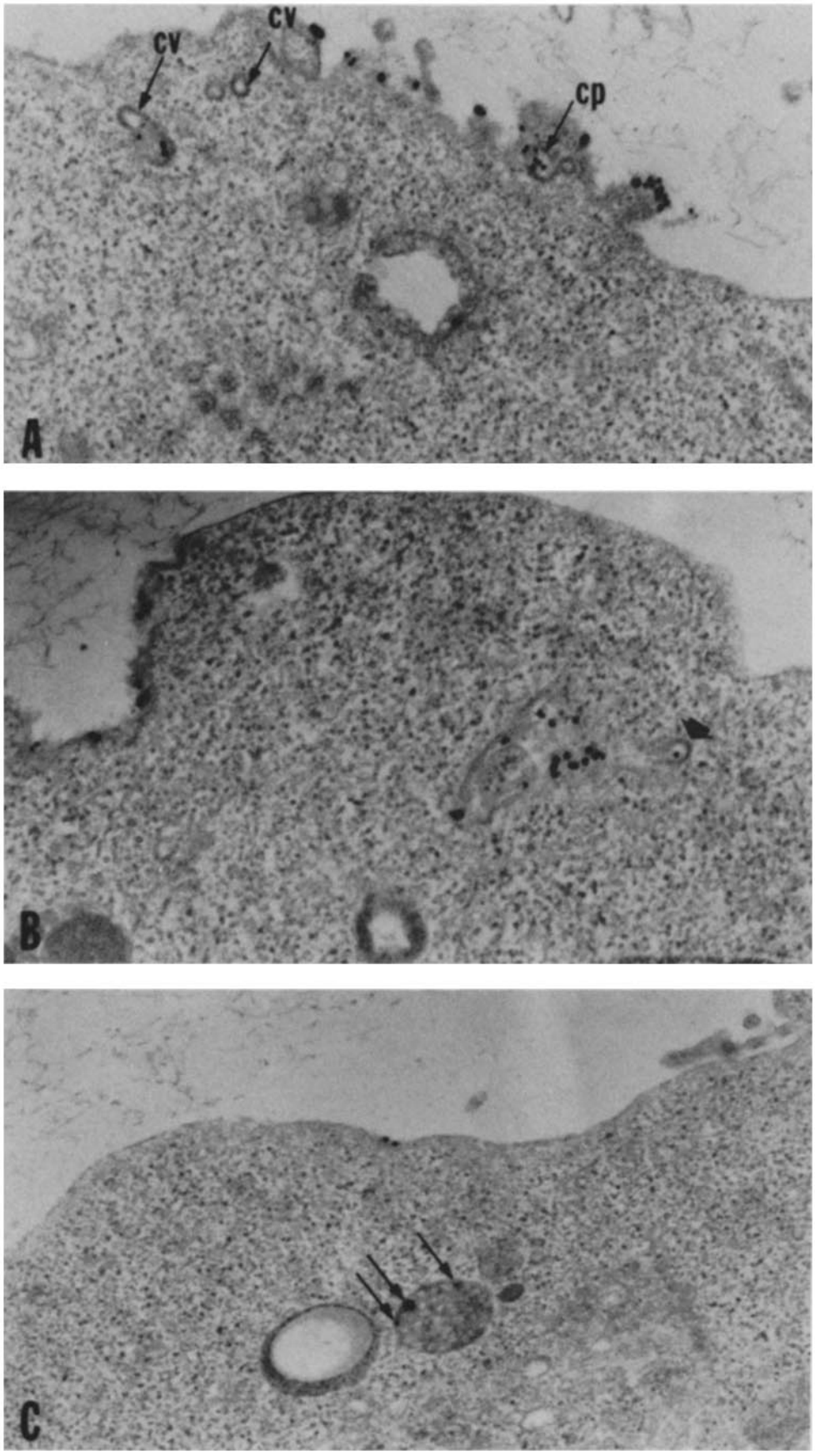

FIGURE 5 The endocytosis of glycophorin by nondifferentiating K562 cells. Cells were labeled at 4°C using glycophorin-detecting antibody followed by a colloidal gold-protein A conjugate. They were then warmed to 37°C for 5 min (*A*), 20 min (*B*), or 1 h (*C*). After 5 min, most gold particles are located at the cell surface in coated pits. At 20 min, particles can be seen in irregular multivesicular structures that interact with coated vesicles (arrow at the far right of the structure in *B*). At 1 h, particles (arrows) are associated with the periphery of multivesicular bodies. *cp*, coated pit; *cv*, coated vesicle profile. Bar, 0.5 μm. × 52,000.

cell after incubation with a polyvalent lectin or antibodies against surface immunoglobulins (4, 24) and, clearly, exclusion of a particular protein from endocytosis would result in its maintenance at the cell surface.

In this report we have shown that the proteins located within clathrin-coated pits change during the differentiation

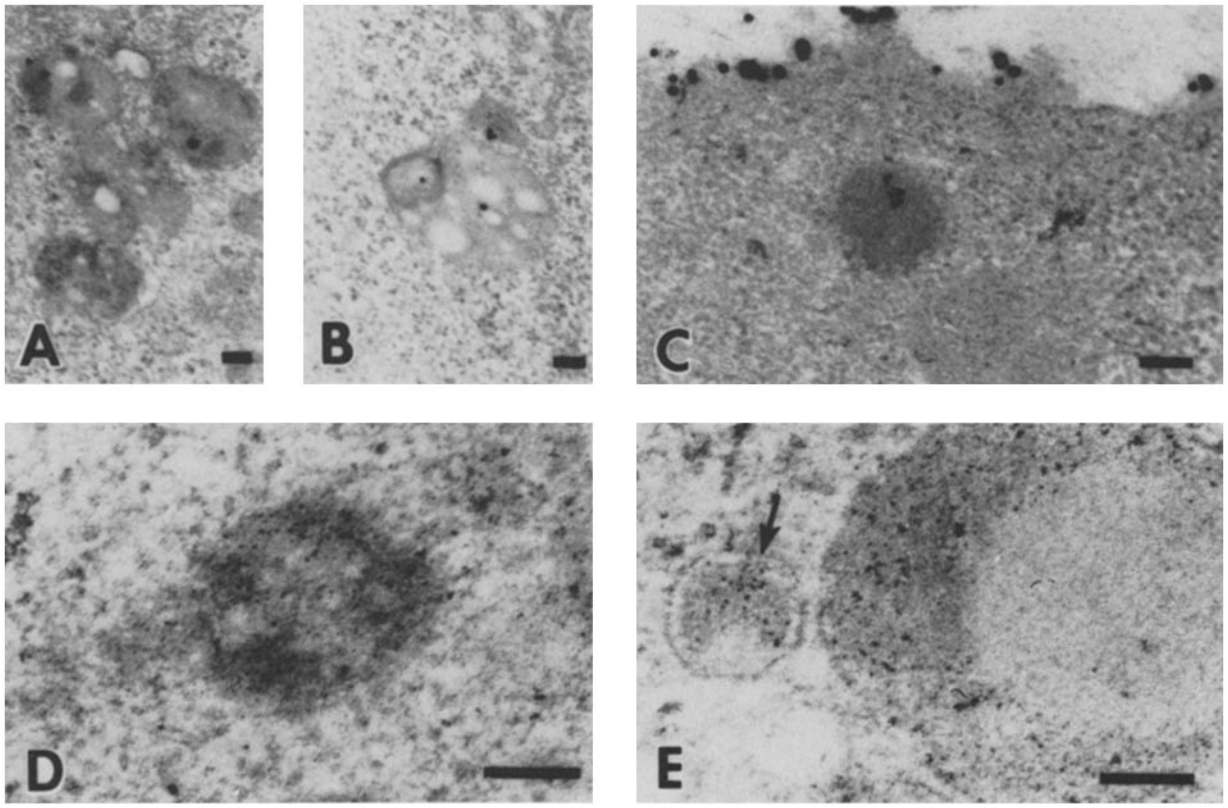

FIGURE 6 The endocytosis of glycophorin and Concanavalin A receptors by K562 cells. Cells were labeled with glycophorin-detecting antiserum and colloidal gold-protein A (*A–C*) or with ferritin-Concanavalin A (*D* and *E*) at 4°C and then warmed to 37°C. (*A* and *B*) Multivesicular bodies from nondifferentiating cells incubated at 37°C for 6 h prior to fixation; (*C*) the surface of a differentiating cell incubated at 37°C for 6 h prior to fixation; (*D*) a lysosome from a nondifferentiating K562 cell labeled with fer-Concanavalin A at 4°C and subsequently incubated at 37°C for 5 min; (*E*) lysosomes from a cell that had been differentiating for 96 h prior to labeling with ferritin-Concanavalin A at 4°C and warming to 37°C for 5 min. The small lysosome arrowed is particularly heavily labeled with ferritin. Bars, 0.1 μm. × 35,000 (*A* and *B*); × 70,000 (*C*); × 125,000 (*D* and *E*).

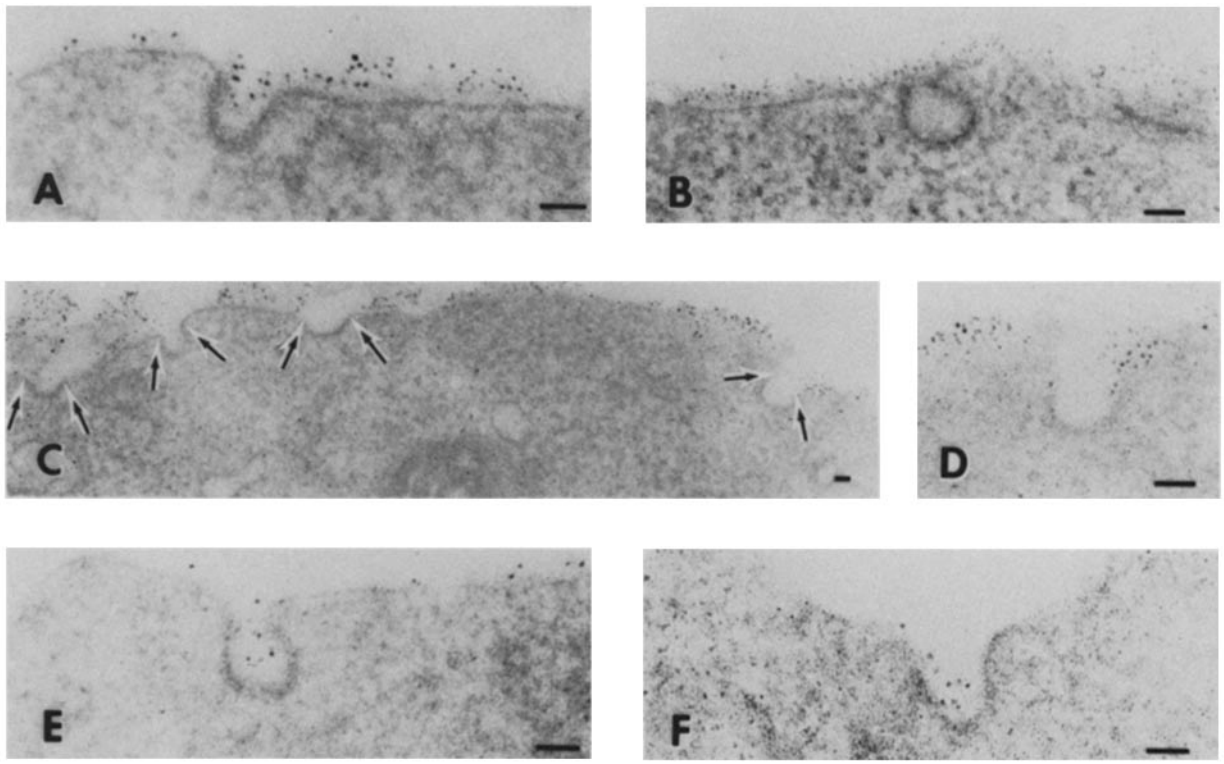

FIGURE 7 Distribution of glycophorin and Concanavalin A receptors on the surfaces of fixed K562 cells. Cells were incubated with either glycophorin-detecting antibody and ferritin-conjugated goat anti-rabbit IgG (*A–D*) or with ferritin Concanavalin A (*E* and *F*) after fixation in glutaraldehyde. (*A*) A coated pit from a nondifferentiating cell. Note that the pit contains ferritin. (*B*) A coated area from a cell that had been differentiating for 96 h. The pit lacks ferritin. (*C*) Several coated areas (delineated by arrows) on the surface of a cell that had been differentiating for 96 h. All lack ferritin. (*D*) A coated area from a cell differentiating for 120 h. Again note the absence of ferritin in the pit. (*E*) A coated area from a nondifferentiating cell. Ferritin-Con A is in the pit. (*F*) A coated area from a cell that had been differentiating for 120 h. Again ferritin-Con A is in the pit. Bars, 0.05 μm. × 130,000 (*A*); × 100,000 (*B*); × 40,000 (*C*); × 110,000 (*D–F*).

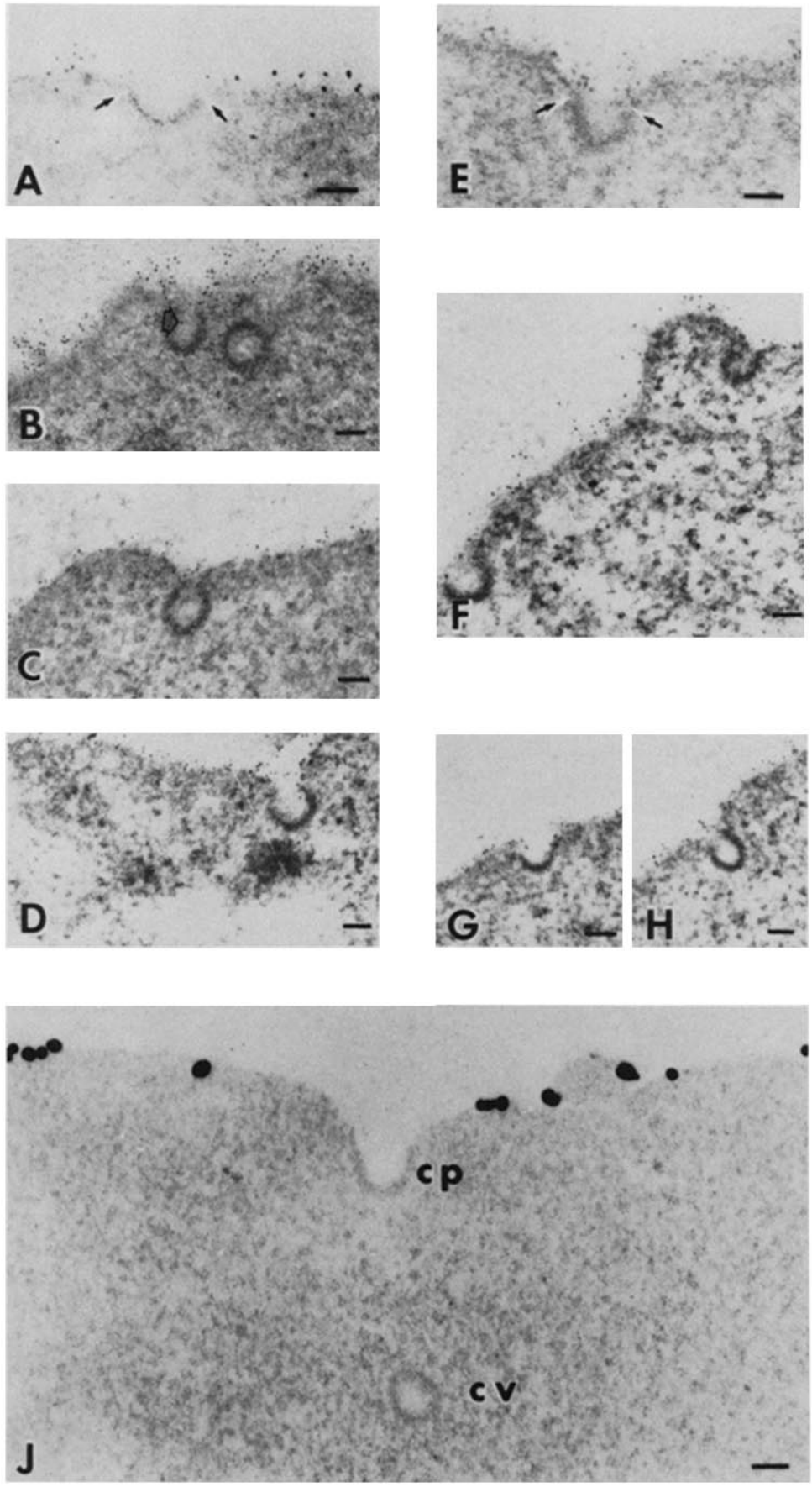

FIGURE 8 The distribution of spectrin on the surface of K562 cells. K562 cells were fixed with glutaraldehyde and then made permeable to antibody with Triton X-100. The cells were then incubated with antispectrin IgG and goat anti-rabbit IgG coupled to ferritin (*A–H*) or with antispectrin IgG and protein A-colloidal gold (*J*). (*A–D*) Coated areas from nondifferentiating cells. In *A* and *E* the coated area is delineated by arrows. A single ferritin particle associated with coated membrane is identified in *B* by an arrowhead. (*E–J*) Coated areas from cells that had been grown for 96 h in hemin-containing medium. *CP*, coated pit; *CV*, coated vesicle profile. Bars, 0.05 μm. × 120,000 (*A* and *E*); × 90,000 (*B–D* and *F–H*); × 110,000 (*J*).

of a human erythroleukemic cell line; in nondifferentiating cells, the clathrin-coated pits incorporate glycophorin which is subsequently endocytosed and delivered to multivesicular bodies. There is, however, little concentration of glycophorin into the coated pits. Because of this lack of concentration of glycophorin into regions of the plasma membrane specialized for endocytosis, antibody-labeled glycophorin molecules are removed from the cell surface only slowly, whereas, in contrast, Con A is rapidly cleared from the cell surface at 37°C because of the preferential association of its receptor with coated pits. After several days of erythroid differentiation, a marked change has occurred in the coated areas of the cell: the coated pits are devoid of any glycophorin, although it remains associated with the noncoated regions of the cell surface. The exclusion of glycophorin from the coated areas of differentiating K562 cells is not the result of a general cessation of the movement of surface glycoproteins into coated areas or of a cessation of endocytosis by coated pits, since Con A receptors are located in the coated pits of both nondifferentiating and differentiating cells and are rapidly endocytosed by both.

In addition to the differentiation-dependent cessation of endocytosis of the external surface glycophorin, spectrin (an erythrocyte peripheral membrane protein on the inner surface of the membrane) appears to be excluded from coated areas by K562 cells; although, in this case the lack of spectrin associated with clathrin-coated areas is not affected by the differentiation state of the cell. This finding is similar to that of Zweig and co-workers (26) who found that reticulocytes from mouse spleen excluded spectrin (as detected by a specific antispectrin antibody) from endocytotic invaginations on the cell surface. Their techniques, however, did not allow them to determine whether these invaginations were clathrin-coated pits.

It is possible that the exclusion of glycophorin from coated pits as differentiation proceeds is related to the distribution of spectrin under the membrane of K562 cells. During differentiation, glycophorin molecules may become less mobile in the plane of the membrane as a result of the induction of an interaction with spectrin, thereby being restrained from entering spectrin-deficient regions of the plasma membrane, namely the coated pits. In this regard it is noteworthy that glycophorin of spectrin-deficient (spherocytic) mouse erythrocytes is more mobile than that of normal erythrocytes (19) and glycophorin mobility in membranes from normal cells may be increased by agents that perturb the spectrin cytoskeleton (20). It therefore appears that as erythroid precursor cells mature in vivo there is a spectrin-dependent restriction of the mobility of integral transmembrane glycoproteins (19) and this may also be the basis of the exclusion of glycophorin from coated pits that we have observed in human erythroleukemic cells.

We thank Mrs. R. Brown for expert typing of the manuscript.

This research was supported by Public Health Service grant CA-26103, American Heart Association (Mississippi Affiliate) grant M-80-G-12 (to R. Hunt), and National Science Foundation grant BNS-77-20220 (to R. and Å. Klein).

Received for publication 20 September 1983, and in revised form 9 January 1984.

REFERENCES

1. Anderson, R. G. W., E. Vasile, R. J. Mello, M. S. Brown, and J. L. Goldstein. 1978. Immunochemical visualization of coated pits and vesicles in human fibroblasts: relation to low density lipoprotein receptor. *Cell.* 15:919–933.
2. Andersson, L. C., M. Jokinen, and C. G. Gahmberg. 1979. Induction of erythroid differentiation in human leukemic cell line K562. *Nature Lond.* 278:364–365.
3. Andersson, L. C., K. Nilsson, and C. G. Gahmberg. 1979. K562—a human erythroleukemic cell line. *Int. J. Cancer.* 23:143–147.
4. Bretscher, M. S., J. N. Thompson, and B. M. F. Pearse. 1980. Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA.* 77:4156–4159.
5. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* 100:119–130.
6. Edelson, P. J., and Z. A. Cohn. 1974. Effects of Concanavalin A on mouse peritoneal macrophages: 1. Stimulation of endocytic activity and inhibition of phagolysosome formation. *J. Exp. Med.* 140:1364–1386.
7. Frazier, J. L., H. J. Caskey, M. Yoffe, and P. A. Seligman. 1982. Studies of the transferrin receptor on both human reticulocytes and nucleated human cells in culture. *J. Clin. Invest.* 69:853–865.
8. Fukuda, M., M. N. Fukuda, and S. Hakomori. 1979. Developmental change and genetic defect in the carbohydrate structure of band 3 glycoprotein of human erythrocyte membrane. *J. Biol. Chem.* 254:3700–3703.
9. Gahmberg, C. G., M. Jokinen, and L. C. Andersson. 1979. Expression of major red cell sialoglycoprotein, glycophorin A, in human leukemic cell line K562. *J. Biol. Chem.* 254:7442–7448.
10. Garvey, J. S., N. E. Cremer, and D. H. Sussdorf. 1977. Methods in Immunology. W. A. Benjamin Inc., Reading, Massachusetts. 223.
11. Geiduschek, J. B., and S. J. Singer. 1979. Molecular changes in the membranes of mouse erythroid cells accompanying differentiation. *Cell.* 16:149–163.
12. Hunt, R. C., and L. M. Marshall. 1981. Membrane protein redistribution during differentiation of cultured human erythroleukemic cells. *Mol. Cell. Biol.* 1:1150–1162.
13. Laemmli, U. K. 1971. Cleavage of structural proteins during assembly of the head of bacteriophage T_4. *Nature Lond.* 227:680–685.
14. Marchesi, V. T. 1974. Isolation of spectrin from erythrocyte membranes. *Methods in Enzymol.* 32:275–277.
15. Marchesi, V. T. 1979. Functional proteins of the human red blood cell membrane. *Semin. Hematol.* 16:3–20.
16. Nunez, M. T., S. Fischer, J. Glass, L. M. Lavidor, E. M. Lenk, and S. H. Robinson. 1977. Transferrin receptors in developing murine erythroid cells. *Brit. J. Haematol.* 36:519–526.
17. Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by use of protein A-gold complex. *J. Histochem. Cytochem.* 26:1074–1081.
18. Rutherford, T., J. B. Clegg, D. R. Higgs, R. W. Jones, J. Thompson, and D. J. Weatherall. 1981. Embryonic erythroid differentiation of the human leukemic cell line K562. *Proc. Natl. Acad. Sci. USA.* 78:348–352.
19. Scheetz, M. P., M. Schindler, and D. E. Koppel. 1980. Lateral mobility of integral membrane proteins. *Nature Lond.* 285:510–512.
20. Schindler, M., D. E. Koppel, and M. P. Scheetz. 1980. Modulation of membrane protein lateral mobility by polyphosphates and polyamines. *Proc. Natl. Acad. Sci. USA.* 77:1457–1461.
21. Segrest, J. P., T. M. Wilkinson, and L. Sheng. 1979. Isolation of glycophorin with deoxycholate. *Biochim. Biophys. Acta.* 554:533–537.
22. Skutelsky, E., and M. G. Farquhar. 1976. Variation in distribution of Con A receptor sites and anionic groups during red blood cell differentiation in the rat. *J. Cell Biol.* 71:218–231.
23. Skutelsky, E., Y. Marikovski, and D. Donen. 1974. Immunoferritin analysis of membrane antigen density. *Eur. J. Immunol.* 4:512–518.
24. Stern, P. L., and M. S. Bretscher. 1979. Capping of exogenous Forssman glycolipid on cells. *J. Cell Biol.* 82:829–833.
25. Turco, S. J., J. S. Rush, and R. A. Laine. 1980. Presence of erythroglycan on human K562 chronic myelogenous leukemia-derived cells. *J. Biol. Chem.* 255:3266–3269.
26. Zweig, S. E., K. T. Tokuyasu, and S. J. Singer. 1981. Membrane-associated changes during erythropoiesis. On the mechanism of maturation of reticulocytes to erythrocytes. *J. Supramol. Struct.* 17:163–181.