Isolation of actin-containing transmembrane complexes from ascites adenocarcinoma sublines having mobile and immobile receptors

(isolated microvilli/microvillar membranes)

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ABSTRACT   The molecular nature of the cell surface–cytoskeleton interaction in microvilli isolated from ascites 13762 rat mammary adenocarcinoma sublines with immobile (MAT-C1) and mobile (MAT-B1) receptors was investigated by extraction and fractionation studies on the microvillar membranes. Extraction of membranes from MAT-C1 cells with Triton X-100-containing buffers gave insoluble residues showing three major components by NaDodSO$_4$/polyacrylamide gel electrophoresis: actin, a 58,000-dalton polypeptide, and a cell surface glycoprotein of 75,000–80,000 daltons. The ratio of these components in Triton X-100-insoluble residues, as determined by scintillation counting of bands from gels of [^{3}H]leucine-labeled microvillar membranes, approached equimolar, suggesting a specific complex of the components. The three components of the putative complex cosedimented on sucrose density gradients of Triton X-100/buffer-treated membranes. Gel filtration on Sepharose 2B gave a peak included in the column that contained only the glycoprotein, actin, and 58,000-dalton polypeptide by one-dimensional NaDodSO$_4$ electrophoresis and by two-dimensional isoelectric focusing/NaDodSO$_4$ electrophoresis. The glycoprotein–actin association could be disrupted only under strongly denaturing conditions. Complex prepared from MAT-B1 microvillar membranes by Sepharose 2B gel filtration in Triton X-100-containing buffers contained actin and the glycoprotein but no 58,000-dalton polypeptide. From these results we propose that the cell surface–cytoskeleton interactions in the 13762 tumor cell microvilli involve direct association of actin with the cell surface glycoprotein. We further suggest that the 58,000-dalton polypeptide stabilizes the association of this complex with the microfilaments in the MAT-C1 microvilli, thereby stabilizing the microvilli and restricting cell surface receptor mobility.

Although the organization of cell surface proteins is widely believed to be controlled by interactions between these surface proteins and a submembrane cytoskeleton (1), via a transmembrane complex (2), there is little information about the molecular nature of these interactions in cells other than erythrocytes. One problem has been the difficulty in obtaining defined cell surface fractions that compare to the erythrocyte membrane (3), the best available model system. Cell surface specializations, such as microvilli, offer obvious advantages in such studies, because they are identified readily by their morphology. Moreover, some cell surface components are concentrated on microvilli (4), suggesting stabilized membrane–cytoskeleton interactions. We have been investigating the nature of membrane–cytoskeleton interactions by using ascites sublines of the 13762 rat mammary adenocarcinoma. The MAT-C1 subline is strikingly different in morphology and cell surface behavior.

MAT-C1 cells have extensively branched microvilli and immobile cell surface receptors. MAT-B1 cells have unbranched microvilli and mobile cell surface receptors (5, 6).

Microvilli from the two sublines have been isolated by a very gentle shearing technique which does not disrupt the cells and which yields morphologically intact (7, 8), sealed (8) microvilli. This preparation of cell surface specializations, uncontaminated by cellular debris, offers a unique plasma membrane fraction with associated cytoskeletal components comparable to erythrocytes in simplicity.

Extraction of MAT-C1 microvilli with Triton X-100 in phosphate-buffered saline at pH 7.4 (P$_i$/NaCl), under conditions in which >90% of the protein and 95% of the carbohydrate is extracted, produced a cytoskeletal residue whose NaDodSO$_4$/polyacrylamide gel electrophoresis profile was quite simple. Coomassie blue staining revealed two major polypeptides in the MAT-C1 microvillar cytoskeletal residue—actin and a 58,000-dalton polypeptide (8). When gels were overloaded or proteins were detected by silver staining, a third major component was detected—a broad band migrating at an apparent molecular mass of 75,000–80,000 daltons (9, 10). By two-dimensional isoelectric focusing/NaDodSO$_4$/polyacrylamide gel electrophoresis (IEF/NaDodSO$_4$ electrophoresis) this component was observed as a multiplet of spots of increasing molecular mass with decreasing pI. Glucosamine labeling and neuraminidase treatments indicated that this component is a glycoprotein (10). The neuraminidase results also indicated a cell surface localization, an observation supported by lactoperoxidase iodination of intact microvilli (10). In addition, DNase treatments (11) and myosin-affinity precipitation analysis (12) showed that the glycoprotein was firmly associated with the microvillar cytoskeleton (10). In light of these results the glycoprotein was termed a cytoskeleton-associated glycoprotein (CAG). However, the mode of association of the glycoprotein with the cytoskeleton remained unclear (9).

We now have extended these studies to investigate the molecular linkage between the membrane and cytoskeleton using membranes isolated from the microvilli by a method that breaks down actin microfilaments (8, 9). By detergent extraction and fractionation studies on MAT-C1 microvillar membranes we have identified and isolated a stable complex of only three components—actin, the 58,000-dalton polypeptide, and CAG. Similar fractionations on microvillar membranes from MAT-B1

cells, whose receptors are mobile, yield a complex containing actin and CAG but no 58,000-dalton polypeptide. These results lead us to propose that interaction between the cell surface and the cytoskeleton in 13762 adenocarcinoma microvilli involves the direct association of actin and CAG. Furthermore, we suggest that the 58,000-dalton polypeptide in MAT-C1 microvilli stabilizes the interaction of the transmembrane complex with the microfilaments in these cells, which have immobile receptors, and in some as yet undefined fashion acts to retard receptor mobility.

MATERIALS AND METHODS

Extraction of Microvilli and Microvillar Membranes. Microvilli or microvillar membranes were isolated from MAT-B1 and MAT-C1 cells or cells labeled metabolically with [^{3}H]leucine as described (8). Detergent extractions of microvilli and membranes were performed in actin-stabilizing buffer (S buffer: 5 mM Tris/0.15 M NaCl/2 mM MgCl$_2$/0.2 mM ATP/0.2 mM dithioerythritol/0.5% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride, pH 7.6) (13) at 25°C for 15 min. Alternatively, extraction in a simpler buffer, Triton X-100 in P$_i$/NaCl, was performed at 37°C for 10 min. In either case, the concentration of microvillar or membrane protein was kept below 150 μg of protein per ml for optimal extraction (8). Samples were chilled on ice and were centrifuged immediately at 100,000 × g for 1 hr to yield microvillar residues or membrane residues.

Sucrose Density Gradient Centrifugation. S buffer extracts of microvillar membranes were centrifuged on gradients of 7–25% sucrose in S buffer for 15 hr at 80,000 × g and 4°C. Fractions were extruded from the top of the tube, dialyzed at 4°C against 0.1% NaDodSO$_4$ containing 0.1 mM phenylmethylsulfonyl fluoride, and lyophilized before dissolving for electrophoresis.

Gel Filtration on Sepharose 2B. Gel filtration was performed on a 0.75 cm × 75 cm Sepharose 2B column preequilibrated with S buffer. Membranes in 1.0 ml of S buffer were chromatographed and 1.2-ml fractions were collected. The fractions were dialyzed and lyophilized as above prior to NaDodSO$_4$ electrophoresis or IEF/NaDodSO$_4$ electrophoresis.

Electrophoresis. One-dimensional NaDodSO$_4$ electrophoresis was performed according to the method of King and Laemmli (14) on 7.5% gels. Two-dimensional IEF/NaDodSO$_4$ electrophoresis was done by the method of Rubin and Milikowski (15), with staining and destaining as previously reported (6, 16). Bands from NaDodSO$_4$ electrophoresis gels of microvillar fractions from cells that were labeled metabolically with [^{3}H]leucine were quantified by excision and extraction for 16 hr at 50°C in Soluene (Packard, Downers Grove, IL), and radioactivity was counted in Insta-Gel.

RESULTS

Extraction of Isolated Microvilli and Microvillar Membranes. To examine the molecular nature of the interaction between CAG and the cytoskeleton, microvilli and microvillar membranes were extracted with nonionic detergent. The comparison between microvilli and microvillar membranes was of particular significance, because microfilaments have been observed in microvilli but not in microvillar membranes, even though actin was observed as the major component of the membranes (8). This actin has been shown to be oligomeric actin (17).

For extractions of microvilli two Triton X-100-containing buffers were compared. S buffer was designated to stabilize actin microfilaments and to retard G- and F-actin interconversions (13). Triton/P$_i$/NaCl is a more commonly used extractant for making cytoskeletal preparations. Fig. 1 (lanes C and D)
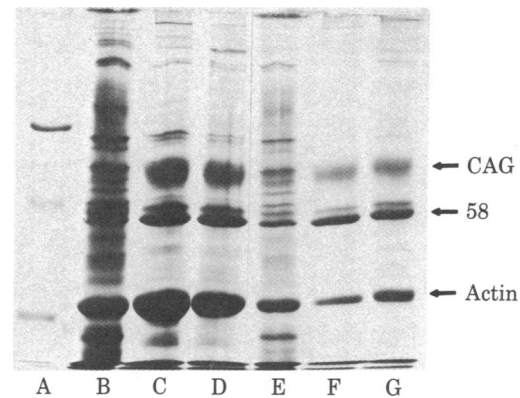

FIG. 1. High-speed pellets of MAT-C1 microvilli (lanes B–D) and membrane fractions (lanes E–G) treated with Triton X-100-containing buffers. Lanes: B, whole microvilli; C, S buffer pellet; D, Triton X-100/P$_i$/NaCl pellet; E, whole membranes; F, S buffer pellet; G, Triton X-100/P$_i$/NaCl pellet. Lane A contains phosphorylase *a* and ovalbumin as standards. The 58,000-dalton polypeptide (58) is indicated.

shows a NaDodSO$_4$ electrophoresis gel of the insoluble residues obtained after extraction of microvilli with each of the extraction buffers. The gels were overloaded deliberately to facilitate observation of the CAG. As previously observed for microvillar residues (9, 10), three major components were present—actin, the 58,000-dalton polypeptide, and CAG (75,000–80,000 daltons). When higher percentage gels were run, no prominent components were observed at molecular masses lower than actin. When the microvillar residues were examined by IEF/NaDodSO$_4$ electrophoresis, actin was observed as two isoelectric species that were identical to those observed for actin isolated from the intact ascites cells (unpublished data) and other nonmuscle cells.

Microvillar membranes were prepared by homogenization of microvilli in glycine/EDTA/2-mercaptoethanol, pH 9.5, buffer (8), conditions which favor disruption and depolymerization of the microvillar microfilaments (8, 17). The same three major components were found in residues after extracting the membranes with S buffer or Triton X-100/P$_i$/NaCl (Fig. 1, lanes F and G). In the S buffer residues other components have been largely extracted. One exception was a polypeptide migrating slightly more slowly than the 58,000-dalton polypeptide. However, because it had the same pI as the 58,000-dalton polypeptide when examined by IEF/NaDodSO$_4$ electrophoresis, it appeared to be a modified form of the 58,000-dalton polypeptide or a very tightly bound component not released by the strong denaturant urea.

Table 1. Ratios of actin to CAG and actin to the 58,000-dalton polypeptide in microvilli, microvillar membranes, and Triton X-100-insoluble residues

Preparation	Actin/CAG	Actin/58,000-dalton polypeptide
Microvilli		
Whole microvilli	ND	18
S buffer residue	7.0	15
Triton X-100/P$_i$/NaCl residue	3.5	7
Microvillar membranes		
Whole membranes	ND	5
S buffer residue	1.3	1.5
Triton X-100/P$_i$/NaCl residue	1.0	1.0

ND, not determined because other bands were present in the CAG region of the gel in unextracted microvilli and membranes.
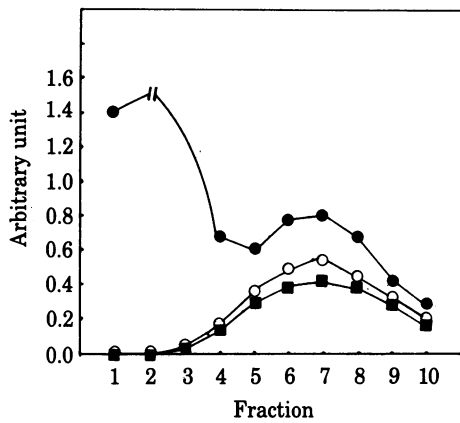
FIG. 2. Cosedimentation profile of microvillar membrane CAG (o), 58,000-dalton polypeptide (■), and actin (●) from 7–25% sucrose density gradient. Quantification was obtained from densitometric traces of NaDodSO$_4$ electrophoresis gel of gradient fractions.

Quantification of the Residue Components. The ratios of the three major components were estimated by excising gel bands of [^{3}H]leucine-labeled samples and determining the radioactivity (Table 1). The amount of actin relative to the other two components was high in microvilli and in the residues obtained after extraction of microvilli with S buffer. These results were expected, because these fractions contain microfilaments (F-actin). Microvillar membranes, in which actin microfilaments are not observed by electron microscopy (8, 17) and which contain largely oligomeric actin (17), contained a lower ratio of actin to the 58,000-dalton polypeptide. CAG cannot be quantified in the unextracted membranes or microvilli because of overlapping membrane polypeptides. Additional actin was removed by detergent extractions of the membranes under conditions used in these experiments, indicating that the membrane-associated (oligomeric) actin was less stable to these extraction conditions than was the F-actin in the microfilaments of the microvilli.

The fact that the ratios among the three components approach equimolar in the membrane residues suggested the presence of a defined complex that might be demonstrated and purified by further fractionations. Because the S buffer membrane residues have few components other than the major three of the putative complex, additional fractionation studies were performed on S buffer extracts of the membranes.

Fractionation of S Buffer Extracts of Microvillar Membranes. To fractionate further the cytoskeletal residue containing the complex, S buffer-solubilized microvillar membranes

from cells metabolically labeled with [^{3}H]leucine were subjected to sucrose density gradient centrifugation on a linear 7–25% gradient. Centrifugation at 80,000 × g for 15 hr at 4°C achieved almost complete separation of CAG and the 58,000-dalton polypeptide from the soluble and smaller components (data not shown), including the MAT ascites cell major glycoproteins ASPG-1 and ASGP-2 (16) and much of the actin. Fig. 2 demonstrates the cosedimentation of CAG, the 58,000-dalton polypeptide, and a portion of the actin, consistent with the specific association of these three components in a complex. The remainder of the membrane actin in the S buffer extract was in the lighter sucrose fractions with the other membrane glycoproteins and polypeptides, as expected from our previous observations on the size of this released actin (17).

To rule out fortuitous cosedimentation of homoaggregates of the three components, [^{3}H]leucine-labeled membranes were solubilized in S buffer and chromatographed on Sepharose 2B. Fractions were collected, dialyzed, lyophilized, and analyzed by NaDodSO$_4$ electrophoresis. Fig. 3 shows CAG, the 58,000-dalton polypeptide, and a portion of the actin eluting within the column near the retention volume of the column (fractions 10–13). Thus, the three components behave as a complex on both sucrose density gradient centrifugation and gel filtration. Samples from the fractions shown in the second and third numbered lanes on the left of Fig. 3 *Right* were pooled, dialyzed, lyophilized, and analyzed by IEF/NaDodSO$_4$ electrophoresis (Fig. 4A). The gel contained only the three major components.

The association of the three components is stable at high pH, low ionic strength, and high ionic strength and appears to be disrupted only by strong denaturants, such as urea, NaDodSO$_4$, and guanidine hydrochloride (unpublished data). These results indicate a stable, specific association among the components of the complex.

Transmembrane Complex from MAT-B1 Cell Microvilli. Because the MAT-B1 subline does not have the 58,000-dalton polypeptide in its microvilli (8), it was of interest to determine whether a complex of actin and CAG could be demonstrated in microvillar membranes from these cells. Therefore, S buffer extracts of MAT-B1 microvillar membranes were subjected to Sepharose 2B gel filtration. The elution profile was very similar to that observed in the analogous experiment for MAT-C1 membranes (Fig. 3 *Left*). The peak containing the complex was examined by IEF/NaDodSO$_4$ electrophoresis and was shown to contain only actin and CAG as significant components (Fig. 4B). These results strongly support the proposal of a membrane complex having a direct interaction between actin and a cell surface glycoprotein.
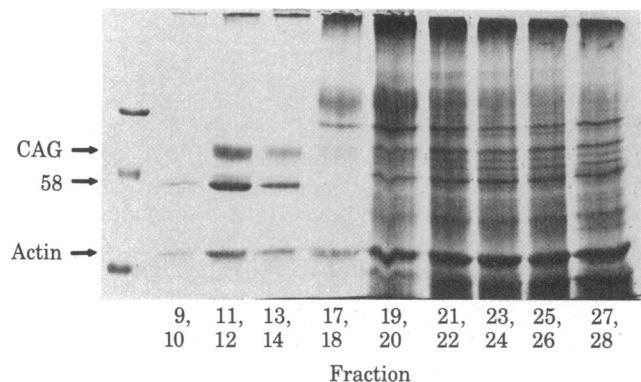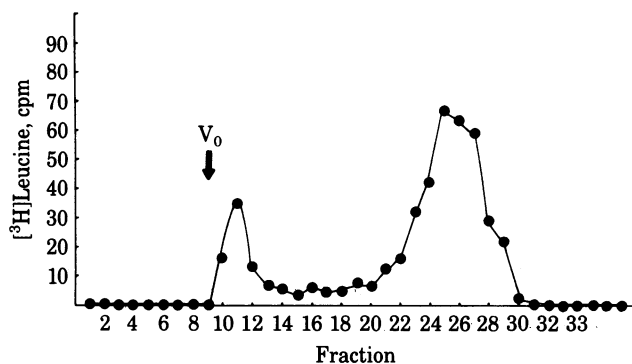

FIG. 3. Sepharose 2B chromatography of MAT-C1 microvillar membranes extracted in S buffer. (*Left*) Elution profile. (*Right*) NaDodSO$_4$ electrophoresis gel of column fractions. The far left lane (not numbered) contains standards of ovalbumin, bovine serum albumin, and phosphorylase *a*. The 58,000-dalton polypeptide (58) is indicated.
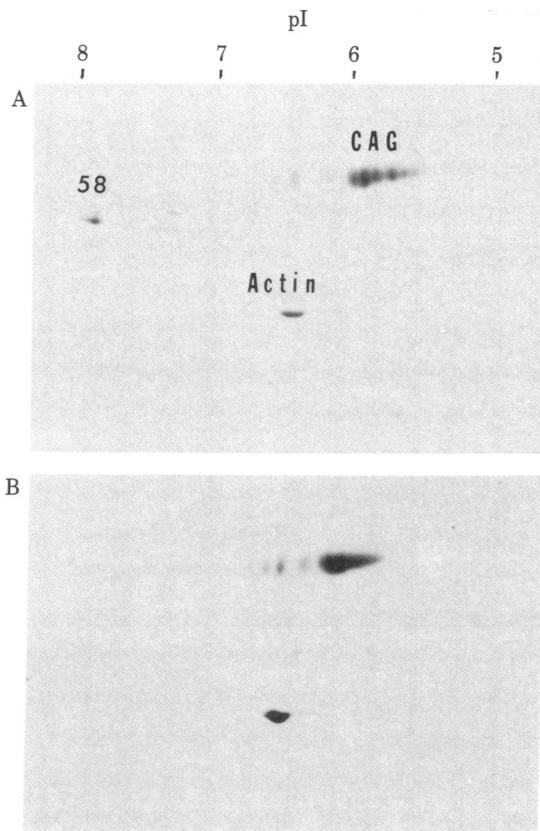
FIG. 4. IEF/NaDodSO$_4$ electrophoresis gels of MAT-C1 (*A*) and MAT-B1 (*B*) isolated complex obtained after Sepharose 2B chromatography.

DISCUSSION

The studies described here provide evidence for a direct linkage between a cell surface molecule and actin. Several lines of evidence indicate that this proposed complex does not result from adventitious associations. (*i*) By metabolic labeling the stoichiometry among the three components is near equimolar. Because we do not know the relative amounts of leucine in each protein or the rates of incorporation of label, it is not possible to predict the exact ratios. However, the fact that the ratios are reproducibly near 1 indicates that this is not simply a nonspecific aggregate. (*ii*) The three components cofractionate on gel filtration and sucrose density gradients. If they were present as three homopolymers, one would expect separation by these techniques unless they were fortuitously all the same size and shape. The sucrose density gradient results indicate that the complex is large and heterogeneous. Estimates by using calibration markers on the gradients or gel filtration columns indicate that the molecular mass values are greater than 10^6 (data not shown). Other membrane components extracted into nonionic detergents show similar behavior—e.g., the acetylcholine receptor (18) and the galactose-specific lectin of liver membranes (19). There are two explanations for this type of behavior. One possibility is that the three component complexes are present in the membrane as multimolecular aggregates, as is the acetylcholine receptor (18). The second possibility is that the complexes are present as discrete units in the membrane, which aggregate upon detergent extraction. Experiments must be undertaken to attempt to distinguish between these alternatives. (*iii*) The actin–CAG association is quite stable to treatments that normally dissociate nonspecific interactions. (*iv*) The actin–CAG complex is found in two ascites sublines with quite different cell surface properties, particularly, cell surface-receptor mobilities (5, 6). The presence of the 58,000-dalton polypeptide as a third member of the complex differs between the two sublines.

We have previously proposed that the restriction of cell surface-receptor mobility and the branched structure of the MAT-C1 microvilli were a consequence of the greater stability of MAT-C1 microvilli (5). This stability can be demonstrated by cytochalasin and hypotonic buffer treatments of the cells (5, 6). The present results provide an explanation for this stability if it is assumed that anchorage of the actin to the membrane determines the stability of the microfilaments, which are necessary to maintain microvillus structure. We propose that microfilaments are linked to the membrane through the actin–CAG association, possibly with an oligomeric actin (17) structure as a linker. We further suggest that *in vivo* the 58,000-dalton polypeptide stabilizes the association of the actin–CAG complex with the microfilaments in the cells to retard disruption of the filament–membrane interaction and thus stabilize the microfilaments and microvilli. Finally, we propose that these cell surface–cytoskeleton interactions provide a restrictive force limiting the mobility of the major cell surface sialoglycoproteins on the MAT-C1 cells. Thus, the CAG–actin interaction may act in a fashion similar to the ankyrin–band 3 system of the erythrocyte membrane (3).

Much more needs to be learned about the interaction of CAG and actin and how this interaction is controlled in the cells. Whether this type of interaction is a general phenomenon is unknown. However, there have been other recent reports of cell surface glycoprotein associations with cytoskeletal residues. H-2 histocompatibility antigen (12) and 5'-nucleotidase (20) remain with a cytoskeletal fraction after nonionic detergent extraction of cell surface membrane fractions, and actin–HLA (human histocompatibility antigen) complexes have been found in detergent extracts (21). In other examples, the association of cell surface glycoproteins with cytoskeletal residues is enhanced by treatment with concanavalin A (22, 23). The mode of association with the cytoskeleton has not been described for any of these examples. Perhaps a direct glycoprotein linkage also occurs in these cases. It is intriguing to speculate that cells may contain several transmembrane glycoproteins with specific cytoplasmic side polypeptide sequences that can bind to actin, thus providing anchorage sites on the cytoskeleton for selected cell surface molecules.

1. Nicolson, G. L. (1976) *Biochim. Biophys. Acta* **457,** 57–108.
2. Bourguignon, L. Y. W. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5031–5035.
3. Branton, D., Cohen, C. M. & Tyler, J. M. (1981) *Cell* **24,** 24–32.
4. de Petris, S. (1978) *Nature (London)* **27,** 66–68.
5. Carraway, K. L., Doss, R. C., Huggins, J. W., Chesnut, R. W. & Carraway, C. A. C. (1979) *J. Cell Biol.* **83,** 529–543.
6. Huggins, J. W., Trenbeath, T. P., Yeltman, D. R. & Carraway, K. L. (1980) *Exp. Cell Res.* **127,** 31–46.
7. Carraway, K. L., Huggins, J. W., Cerra, R. F., Yeltman, D. R. & Carraway, C. A. C. (1980) *Nature (London)* **285,** 508–510.
8. Carraway, C. A. C., Cerra, R. F., Bell, P. B. & Carraway, K. L. (1982) *Biochim. Biophys. Acta* **719,** 126–139.
9. Carraway, K. L., Huggins, J. W., Sherblom, A. P., Howard, S. C., Hull, S. R., Cerra, R. F., Jung, G. & Carraway, C. A. C. (1982) in *Membranes in Tumour Growth,* eds. Galeotti, T., Cittadini, A., Neri, G. & Papa, S. (Elsevier/North-Holland, Amsterdam), pp. 205–212.

10. Carraway, C. A. C., Jung, G., Craik, J. R., Rubin, R. W. & Carraway, K. L. (1983) *Exp. Cell Res.* **143**, 301–306.
11. Sheetz, M. P. (1979) *J. Cell Biol.* **81**, 266–277.
12. Koch, G. L. E. & Smith, C. R. (1978) *Nature (London)* **273**, 274–278.
13. Strauch, A. R., Luna, E. J. & La Fountain, J. R., Jr. (1980) *J. Cell Biol.* **86**, 315–325.
14. King, J. & Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465–471.
15. Rubin, R. W. & Milikowski, C. (1978) *Biochim. Biophys. Acta* **509**, 100–110.
16. Sherblom, A. P. & Carraway, K. L. (1980) *J. Biol. Chem.* **255**, 12051–12059.
17. Carraway, K. L., Cerra, R. F., Jung, G. & Carraway, C. A. C. (1982) *J. Cell Biol.* **94**, 624–630.
18. Heidmann, T. & Changeux, J. P. (1978) *Annu. Rev. Biochem.* **47**, 317–357.
19. Neufeld, E. F. & Ashwell, G. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans*, ed. Lennarz, W. J. (Plenum, New York), pp. 241–266.
20. Mescher, M. F., Jose, M. J. L. & Balk, S. P. (1981) *Nature (London)* **289**, 139–144.
21. Pober, J. S., Guild, B. C., Strominger, J. L. & Veatch, W. R. (1981) *Biochemistry* **20**, 5625–5633.
22. Sheterline, P. & Hopkins, C. R. (1981) *J. Cell Biol.* **90**, 743–754.
23. Painter, R. G. & Ginsberg, M. (1982) *J. Cell Biol.* **92**, 565–573.