Journal of Supramolecular Structure
and Cellular Biochemistry 16:105–113 (1981)
Mechanisms of Chemical Carcinogenesis 31–39

# Genetics of Receptors for Bioactive Polypeptides: A Variant of Swiss/3T3 Fibroblasts Resistant to a Cytotoxic Insulin Accumulates Lysosome-Like Vesicles

**W. Keith Miskimins, Wayne R. Ferris, and Nobuyoshi Shimizu**

*Department of Cellular and Developmental Biology, University of Arizona, Tucson, Arizona 85721*

Recently we have isolated six variants of Swiss/3T3 mouse fibroblasts that are resistant to the cytotoxic insulin-diphtheria toxin A fragment. All of the variants proved to have greatly reduced or no insulin binding capacity, and several variants showed altered morphologies and growth characteristics. We now report on the further characterization of one of these variants, CI-3, which displays a massive accumulation of membranous vesicles in its cytoplasm. By electron microscopy these vesicles resemble lysosomes. They also appear to fluoresce bright orange after treatment of viable cells with acridine orange. However, the specific activity of several lysosomal enzymes is depressed in CI-3. Additionally, there is an apparent shift in the density of vesicles containing lysosomal enzymes in this variant. These alterations may be directly related to CI-3's resistance to the cytotoxic insulin and have some important bearings on the mechanism of insulin action.

**Key words: hormone receptors, diphtheria toxin, lysosomes, hybrid proteins**

To study the interaction of polypeptide hormones and growth factors with their cell surface receptors and the subsequent production of a biological response, we have devised a means by which to select relevant genetic variants [1, 2]. As a selection agent, insulin was cross-linked to the enzymatically active fragment A of diphtheria toxin [1]. This conjugate was shown to kill insulin-responsive cells through an insulin receptor-dependent mechanism [1]. When this hybrid molecule was utilized for the isolation of resistant Swiss/3T3 mouse fibroblasts, several variant colonies were obtained [3]. Each of these colonies proved to have a reduced insulin-binding capacity. Two of the isolates were further characterized [3] and shown to have altered morphologies and growth kinetics, but to retain epidermal growth factor binding.

We now describe a more detailed characterization of one of the insulin-fragment A resistant variants, CI-3. This cell line displays some unique qualities that may be directly related to the mechanism of insulin action. The results further demonstrate the utility of cytotoxic hybrid molecules for the selection of genetic variants and the use of these variants in elucidating the molecular mechanisms of action of bioactive polypeptides.

Received February 24, 1981; accepted April 14, 1981.

**0275-3723/81/1601-0105$03.00** 

## MATERIALS AND METHODS

### Culture Conditions

The cell lines were grown in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Electron Microscopy

The cells were harvested and pelletted by centrifugation at 500 rpm. The pellet was then fixed for 3 hr in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature. After a thorough rinsing in buffer, the pellet was post-fixed for 1 hr at 4°C in 1% $OsO_4$ in 0.1 M cacodylate buffer. Embedding was in Epon 812. Sections were cut with a Sorvall MT-2 microtome and observed in either a Phillips EM200 or a JEOL EM 100CX electron microscopy.

### Acridine Orange Staining

Cells were grown on round polystyrene coverslips (25 mm). For staining [4], acridine orange (Sigma) was added directly to the culture media to a final concentration of 10 μg/ml. The cells were then incubated in the dark for 30 min at 37°C. At the end of this incubation period, the cells were rinsed with phosphate-buffered saline (PBS) in low light. After an additional 15 min at 37° in fresh medium, the coverslips were rinsed and mounted cell side up on a microscope slide. A drop of PBS was added, and a glass coverslip was applied. The cells were then viewed using a Nikon Fluophot fluorescent microscope equipped with epi-illumination using an IF420-490 excitation filter, DM505 dichroic mirror, and 520W eyepiece-side absorption filter. Photo micrographs were taken using Kodak Ektochrome 200 film with a 15-second exposure.

### Enzyme Analysis

β-Galactosidase, β-hexosaminidase, β-glucuronidase, and acid phosphatase were all assayed using 4-methylumbelliferyl derivatives (Sigma) as substrates [5]. The reaction mixtures (0.4 ml) contained 0.1 M sodium acetate, 0.1 M Triton X-100, 1 mM 4-methylumbelliferyl substrates, 150 μg/ml protein from 5–50 μl of cell extract (prepared according to [6]) or Percoll gradient fraction. The reaction was initiated with the addition of substrate and carried out at 37°C for the designated times. The reaction was stopped with 2 ml of 0.5 M $Na_2CO_3$. Fluorescence was read using a model 111 Turner fluorometer with a 7–60 primary filter and a 2A secondary filter. Activity is given as a relative fluorescence.

### Percoll Gradient Centrifugation

Cells were washed once in PBS and once in 0.25 M sucrose and pelletted by centrifugation at 500 rpm. The cell pellet was resuspended in 1 ml of 0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, and 10 mM triethanolamine, pH 7.4 (SEAT buffer). Lysis was achieved by repetitively pipetting (20 times) with a 1-ml pipette [7]. The lysate was centrifuged at 800 rpm for 10 min. The supernatant was collected, and the pellet was resuspended in 1 ml of the same buf-

fer. Lysis and centrifugation were repeated and the two supernatants combined. This supernatant was then centrifuged at 20,000g for 20 min. The resulting pellet was resuspended in 0.5 ml of the same buffer and gently layered on the top of a 9.5 ml suspension of Percoll (Pharmacia Fine Chemicals) in a polyallomer centrifuge tube. The suspension consisted of Percoll, SEAT buffer, and 2.5 M sucrose in a 9:16:1 volume ratio. The tube was then centrifuged at 30,000g for 60 min in a Sorvall SS34 rotor at 4°C. Fractions (0.3 ml) were collected by dripping from the bottom of the tube.

## RESULTS

CI-3 is a clonal cell line derived from Swiss/3T3 mouse fibroblasts on the basis of its resistance to the killing action of insulin cross-linked to the active fragment A of diphtheria toxin [3]. Previously we demonstrated that this variant has a greatly reduced insulin binding capacity as well as altered growth kinetics [3]. In addition, the morphology of this cell line, as revealed by light microscopy, was notably different from that of the parental 3T3 cells. We therefore examined this variant using electron microscopy. The results are shown in Figure 1. There is a striking accumulation of membrane-bound vesicles with a dense appearance. These bodies are highly polymorphic in structure and content, taking on a variety of shapes and sizes and often appearing to enclose lamellar structures. Although a definite identification would require cytochemical analysis, these vesicles resemble lysosomes, and most probably represent residual bodies arising from autophagy and secondary lysosomes [8, 9]. Similar structures were found only rarely in the parental Swiss/3T3 cells.

Additional evidence that the vesicles amassed in CI-3 are components of the lysosomal apparatus comes from vital staining with acridine orange. This fluorescent probe has been shown specifically to label lysosomes in living cells [4, 10]. At 37°C acridine orange is taken up and concentrated in the lysosomes where, in this environment, it emits a bright orange color, Nuclei fluoresce green. Figure 2 compares acridine orange-treated CI-3 cells with identically treated Swiss/3T3 cells. Numerous fluorescent spots are evident throughout the cytoplasm of CI-3 cells (Fig. 2A). In contrast, the fluorescent spots are much sparser in Swiss/3T3 cells (Fig. 2B). Since both electronmicroscopy and vital fluorescent staining indicated a massive development of lysosome-like bodies in CI-3, we measured the activity of several lysosomal acid hydrolases in extracts of these cells. Unexpectedly, as shown in Figure 3, the specific activities of all three hydrolases examined ($\beta$-hexosaminidase, $\beta$-galactosidase, and $\beta$-glucuronidase) were decreased in CI-3 when compared to the parental 3T3 cells. Because of this apparent conflict between the cytological and enzyme activity data, we undertook to characterize the vesicles biochemically. This was done by separation of the subcellular particles on a Percoll density gradient [11, 12]. The position of lysosomes or vesicles was followed by assaying $\beta$-glucuronidase activity. As shown in Figure 4, both CI-3 and Swiss/3T3 produced two major peaks of lysosomal enzyme activity in the density gradient. The more buoyant peak sedimented only a few fractions from the top of the gradient, at an identical position for CI-3 and the parental cell line. This peak may include a type of lysosome or an extra lysosomal distribution of

Fig 1 Electron micrographs showing the morphology of lysosome-like vesicles which accumulate in CI-3 (A) as compared to Swiss/3T3 (B) Bar represents 1 $\mu$

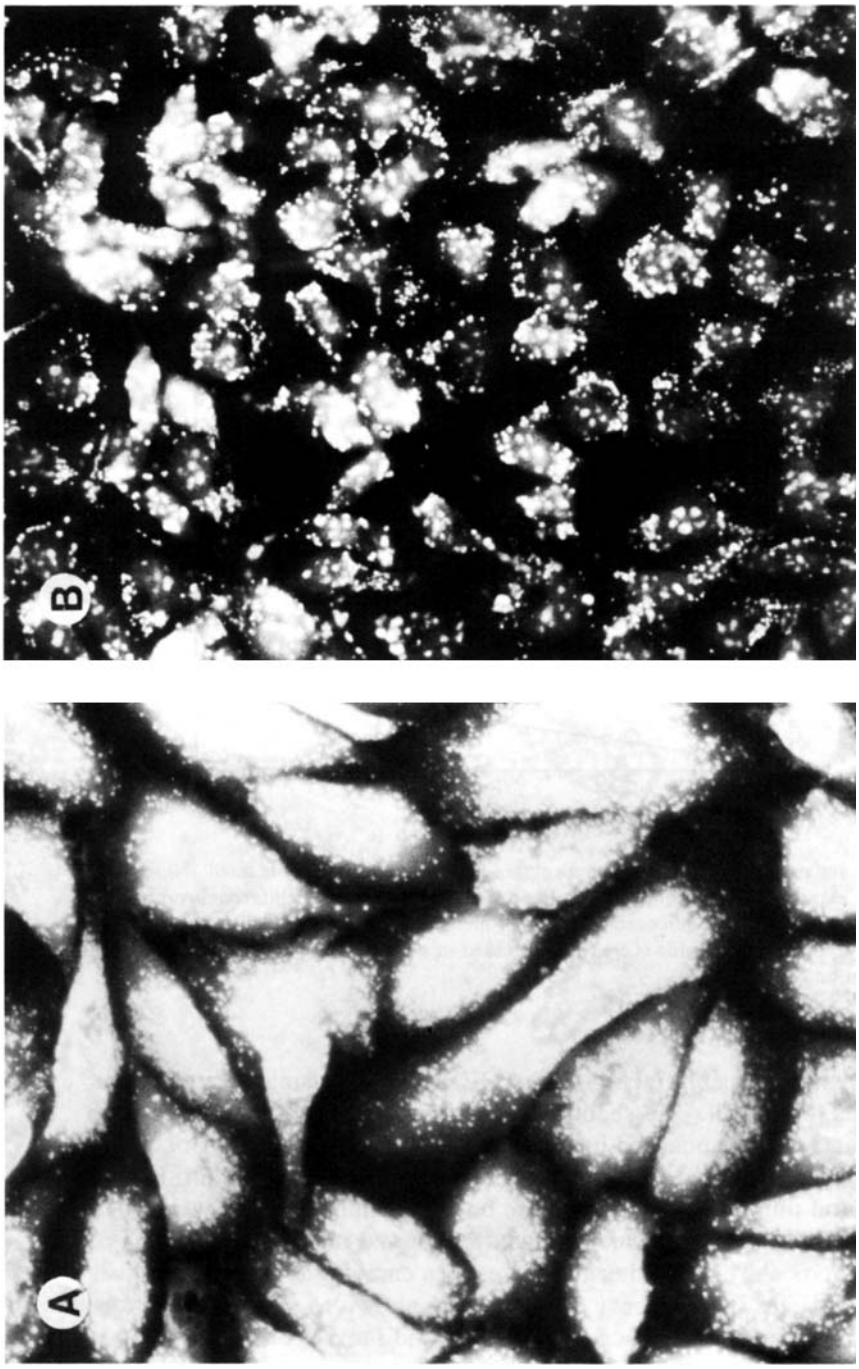


Fig 2 Acridine orange staining of variant and parental cells Confluent Cl-3 cells (A) and Swiss/3T3 cells (B) were incubated for 30 min at 37°C in the presence of acridine orange, washed free of acridine orange, and incubated for an additional 15 min The cells were then observed by fluorescence microscopy The lysosomes appeared as pinpoint spots of fluorescence in the cytoplasm

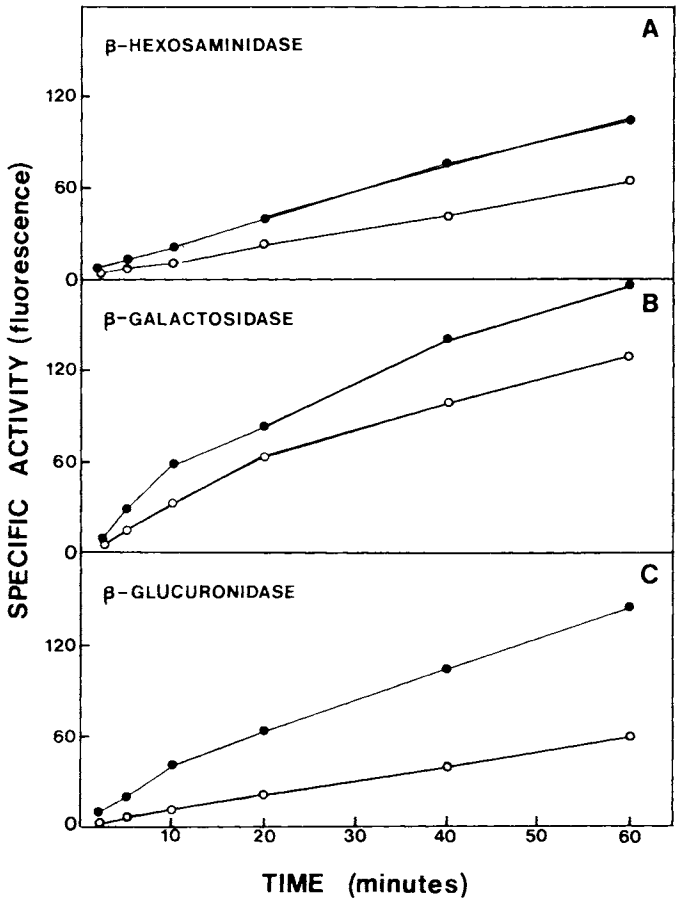


Fig. 3. Specific activity of lysosomal acid hydrolases. Reaction mixtures contained 150 μg of protein from cell extracts. Fluorescence due to the release of 4-methylumbelliferone from 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (A), 4-methylumbelliferyl-β-D-galactoside (B), or 4-methylumbelliferyl-β-D-glucuronide (C) was determined in a model 111 Turner fluorometer. Swiss/3T3 (—•—) and CI-3 (—○—).

lysosomal enzymes, possibly including the GERL, endoplasmic reticulum, or Golgi apparatus [5, 13–15]. The second, denser peak of activity, however, did not sediment at the same position in the gradient in both cell lines. For Swiss/3T3, this peak of activity was found near the midpoint of the gradient, but for CI-3 it was found only 5 fractions from the bottom. Similar results were obtained when acid phosphatase was used as a lysosomal marker. Thus in CI-3 there is an obvious shift in the density of vesicles carrying lysosomal hydrolases. The vesicles in this dense fraction were shown to produce an orange fluorescence in the presence of acridine orange, and most probably correspond to the numerous bodies observed by electronmicroscopy.

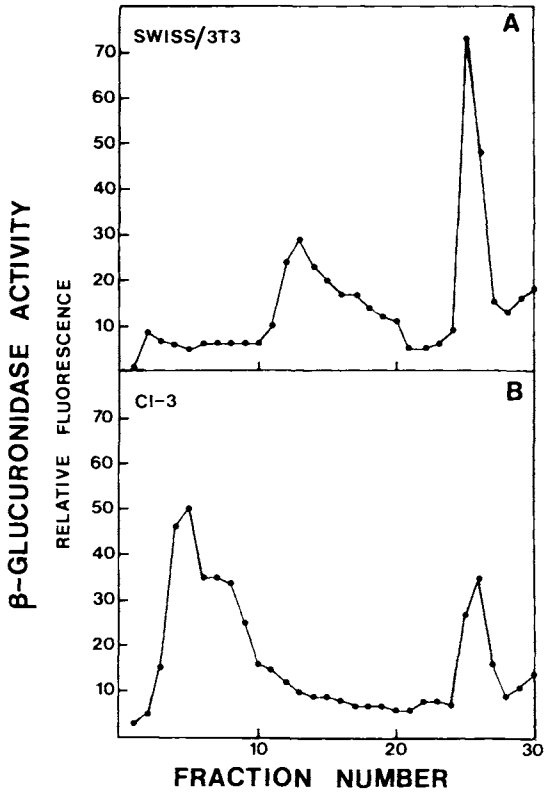


Fig. 4. Percoll density gradient analysis of subcellular fractions. Swiss/3T3 cells (from a 100-mm culture dish, A) and CI-3 cells (from a 150-mm culture dish, B) were gently lysed, nuclei were removed, and the 20,000-g particulate was layered on a suspension of Percoll. A density gradient was developed by centrifuging at 30,000g for 60 min. Fractions were collected and, as a lysosomal marker, the β-glucuronidase activity from each fraction was determined.

## DISCUSSION

The cell line, CI-3, studied in this report is a variant of Swiss/3T3 mouse fibroblasts, which survives in the presence of the cytotoxic insulin-diphtheria toxin fragment A conjugate [3]. The killing action of this conjugate is dependent upon functional insulin receptors and the ability of fragment A to reach the cytoplasmic compartment, where it can enzymatically inhibit protein synthesis by inactivating elongation factor 2 [1, 14]. Therefore, several types of genetic lesions may render a cell resistant to this hybrid molecule. Some obvious possibilities are a defective insulin receptor that cannot bind insulin, or a receptor that binds but cannot internalize insulin. Previously, we demonstrated that CI-3 retains only about 10% of the parental 3T3 cell's insulin-binding capacity [3]. Additionally, we found that CI-3 has a reduced growth rate and obvious morphological alterations. The ultrastructure of this variant was therefore exam-

ined. We found (Fig. 1) an extensive accumulation of electron-dense, polymorphic, lysosome-like vesicles in its cytoplasm. The lysosomal nature of these vesicles was further demonstrated by vital staining with acridine orange (Fig. 2). However, when the specific activities of typical lysosomal enzymes were assayed, they were found to be depressed in CI-3 in comparison to Swiss/3T3 (Fig. 3). In addition, when the cells were fractionated in a Percoll density gradient, there was found to be a dramatic shift in the density of the less bouyant peak of vesicles containing lysosomal enzyme activity (Fig. 4). Thus, there is an apparent alteration in the vacuolar apparatus [8, 15] in this insulin-fragment A resistant variant.

It is known that insulin plays a role in the regulation of intracellular protein breakdown [16–21]. The major effect of insulin is the inhibition of a proteolytic system, which can be induced by glucagon, amino acid deprivation, serum starvation, and other stressful conditions and involves cellular autophagy [22–26]. The induction of this autophagic–lysosomal system of intracellular protein breakdown is associated with the appearance of cytoplasmic vesicles, which are morphologically similar to those in CI-3, and a large increase in turnover of cellular proteins without an increase in the activities of lysosomal enzymes [25]. Additionally, a striking increase in the size and number of hepatic autophagic vacuoles and dense bodies has been demonstrated in diabetic rats [27, 28]. This effect was completely reversible by the administration of insulin [27, 28]. In light of these findings, one possibility is that the alteration in CI-3 that enables it to survive in the presence of the insulin-fragment A conjugate leads to decreased insulin binding, and the inability to produce the signal by which it regulates cellular protein turnover. In this regard, we are studying the lysosomal proteinase activity and turnover of cellular proteins in this variant.

Recently, Sandvig and Olsnes [29] and Draper and Simon [30] have demonstrated a role for lysosomal processing in the mechanism of intact diphtheria toxin action. These workers propose that cell-bound toxin is endocytosed and delivered to the lysosomal compartment. The low pH of this environment then triggers the opening of channels through which fragment A is inserted through the membrane into the cytoplasm. It has also been demonstrated that insulin is delivered to the lysosomal compartment through receptor-mediated endocytosis [31–34]. The toxicity of our insulin-diphtheria toxin fragment A may therefore also depend on a lysosomal pathway in a manner similar to that of intact toxin. The abnormality observed in the lysosomal system of CI-3 may further contribute to this cell line's resistance to the conjugate. For example, CI-3 may possess the inability to form a channel in the membrane or to split the insulin-fragment A conjugate, both of which may be necessary for toxicity. This may in turn suggest an important role for this pathway in the mechanism of insulin action.

In view of the recent evidence that many polypeptide hormones and growth factors are internalized and processed through a lysosomal pathway [33–41], CI-3 offers a unique system in which to study this phenomenon. We are currently approaching this problem using epidermal growth factor since CI-3 cells possess its receptors, and hope to gain some knowledge of this pathway's relationship to the mitogenic action of these hormones.

## ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant GM24375. N. Shimizu is a recipient of American Cancer Society Junior Faculty Research Award JFRA-9.

## REFERENCES

1 Miskimins WK, Shimizu N Biochem Biophys Res Commun 91 143, 1979
2 Shimizu N, Miskimins WK, Shimizu Y FEBS Lett 118 274, 1980
3 Miskimins WK, Shimizu N Proc Natl Acad Sci USA 78 445, 1981
4 Poole AR In Dingle JT (ed) "Lysosomes, a Laboratory Handbook" Amsterdam North-Holland, 1977, pp 313–316
5 Rome LH, Garvin AJ, Allietta MM, Neufeld EF Cell 17 143, 1979
6 Shimizu N, Behzadian MA, Shimizu Y Proc Natl Acad Sci USA 77 3600, 1980
7 Harms E, Kern H, Schneider JA Proc Natl Acad Sci USA 77 6139, 1980
8 De Duve C, Wattiaux R Ann Rev Physiol 28 435, 1966
9 Novikoff AB In Hers HG, Van Hoof F (eds) "Lysosomes and Storage Diseases" New York Academic Press, 1973, pp 1–41
10 Allison AC, Young MR In Dingle JT, Fell HB (eds) Lysosomes in Biology and Pathology" Amsterdam North-Holland, 1969, pp 601–628
11 Wolff DA In Prescott DM (ed) 'Methods in Cell Biology" New York Academic Press, 1975, pp 85–104
12 Pertoft H, Laurent TC In Catsimpoolas N (ed) 'Methods in Cell Separation" New York Plenum, 1977, pp 25–65
13 Pertoft H, Warmegard B, Hook M Biochem J 174 309, 1978
14 Harms E, Kartenbeck J, Darai G, Schneider J Exp Cell Res 131 251, 1981
15 Gelman BB, Davis MH, Morris RE, Gruenstein E J Cell Biol 88 329, 1981
16 Pappenheimer AM Jr Ann Rev Biochem 46 69, 1977
17 De Duve C In Dingle, JT, Fell HG (eds) Lysosomes in Biology and Pathology," Vol 1 Amsterdam North-Holland, 1969, pp 3–40
18 Hershko AP, Mamont RS, Tomkins GM Nature New Biol 232 206, 1971
19 Gelehrter TD, Emanuel JR Endocrinology 94 676, 1974
20 Amenta JS, Sargus MJ, Baccino FM Biochem J 168 223, 1977
21 Gunn JM, Clark MG, Knowles SE, Hopgood MF, Ballard FJ Nature 266 58, 1977
22 Ballard FJ, Wong SSC, Knowles SE, Partridge NC, Martin RJ, Wood CM, Gunn JM J Cell Physiol 105 335, 1980
23 Mortimore GE, Mondon CE J Biol Chem 245 2375, 1970
24 Ashford TP, Porter KR J Cell Biol 12 198, 1962
25 Deter P, De Duve C J Cell Biol 33 347, 1967
26 Mortimore GE, Schworer CM Nature 270 174, 1977
27 Amenta JS, Sargus MJ, Venkatesan S, Shinozuka H J Cell Physiol 94 77, 1978
28 Amenta JS, Brocher SC Life Sci 28 1195, 1981
29 Amherdt M, Harris V, Renold AE, Orci L, Unger RH J Clin Invest 54 188, 1974
30 Pain VM, Lanoix J, Bergeron JJM, Clemens MJ Biochim Biophys Acta 353 487, 1974
31 Sandvig K, Olsnes S J Cell Biol 87 828, 1980
32 Draper RK, Simon MI J Cell Biol 87 849, 1980
33 Gorden P, Carpentier J-L, Freychet P, Le Cam A, Orci L Science 200 782, 1978
34 Carpentier J-L, Gorden P, Barazzone P, Freychet P, Le Cam A, Orci L Proc Natl Acad Sci USA 76 2803, 1979
35 Schlessinger J, Schechter Y, Willingham MC, Pastan I Proc Natl Acad Sci USA 75 2659, 1978
36 Marshall S, Olefsky JM J Biol Chem 254 10153, 1979
37 Carpenter G, Cohen S J Cell Biol 71 2644, 1976
38 Haigler HT, McKanna JA, Cohen S J Cell Biol 81 382, 1979
39 Fox CF, Das M J Supramol Struct 10 199, 1979
40 Ascoli M, Puett D J Biol Chem 253 4892, 1978
41 Goldstein JL, Brown MS Ann Rev Biochem 46 897, 1977