STUDIES OF INTRAORGANELLE DYNAMICS: THE LYSOSOME, THE PRE-LYSOSOMAL COMPARTMENT, AND THE GOLGI APPARATUS

by

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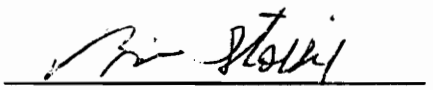

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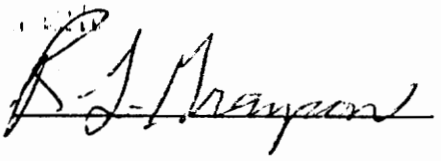

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(ABSTRACT)

The lysosome, a multi-copy organelle, was chosen as an example to study intraorganelle dynamics. Lysosomal contents and membrane proteins were shown to intermix rapidly in fused mammalian cells, with a $t_{1/2}$ of ~30 min. Lysosomal content intermixing, shown by a sensitive invertase-lysosome/[^{14}C]-sucrose-lysosome pairing assay, was inhibited greatly by ATP inhibitors and partially by cytochalasin D. Lysosomal membrane protein intermixing was shown by the transfer of LAMP-2, a mouse specific lysosomal membrane antigen, from mouse lysosomes to hamster sucrosomes, sucrose-swollen lysosomes. Lysosomal membrane protein intermixing was also shown by the co-localization of LIMP I, a rat specific lysosomal membrane antigen, and LAMP-1, a mouse specific lysosomal membrane antigen. Co-localization was assessed by both double immunofluorescent staining and double immunogold labeling of thin cryosections. Both lysosomal content and membrane protein intermixing were inhibited by nocodazole, a microtubule disrupter. In fused cells, lysosomes remained small, punctate and scattered throughout the cytoplasm. In comparison to lysosomes, the prelysosomal compartment (PLC), a single copy orga-

nelle which is related to the lysosome, congregated together to form an extended PLC complex associated with clustered nuclei.

The intermixing of both resident and transient Golgi membrane proteins was studied in fused cells. Resident Golgi membrane protein intermixing was slow, with a $t_{1/2}$ of ~ 1.75 h; it was concomitant with the congregation of the Golgi units. In comparison, the transient Golgi membrane protein was transported much faster from Golgi units to the other Golgi units, with the $t_{1/2} \leq 15$ min. Transient Golgi membrane protein transport occurred between separate Golgi units. These results are consistent with two different pathways for resident and transient Golgi membrane protein transport: a slow, lateral diffusion along the Golgi connections transport pathway for resident Golgi membrane proteins; and a rapid, transient protein selective, vesicle-mediated transport pathway for transient Golgi membrane proteins.

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List of Abbreviations

BSA, Bovine serum albumin

CHO, Chinese ovary hamster

DAB, 3,3'-diaminobenzidine

FITC, fluorescein isothiocynate

$GIMP_c$, Golgi integral membrane protein located at the cis-cisternae

Golgp125, Golgi glycoprotein, 125 kDa

HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HRP, horseradish peroxidase

LAMP, lysosomal associated membrane protein; mLAMP, mouse LAMP; hLAMP, human LAMP

lgp, lysosomal glycoprotein

LIMP, lysosomal integral membrane protein

MES, 2[N-morpholino]ethanesulfonic acid

MPR, mannose 6-phosphate receptor; CI MPR, cation dependent MPR.

NEM, N-ethylmaleimide

NSF, N-ethylmileimide sensitive fusion protein

NRK, normal rat kidney

PBS, Dulbecco's phosphate-buffered saline

PEG, polyethylene glycol

PLC, pre-lysosomal compartment

PMA, phorbol myristate acetate

VSV, vesicular stomatitis virus; VSV G, VSV glycoprotein

Chapter 1. General Introduction

1.1. General organelle dynamics: interorganelle versus intraorganelle

Eukaryotic cells consist of a variety of different subcellular compartments, namely organelles. Some organelles are single-copy per cell, like the nucleus and the Golgi apparatus, and others are multi-copy per cell, like the lysosome and the mitochondrion. Different organelles are structurally and functionally distinct. However, they are also interrelated to each other. For example, the endoplasmic reticulum and Golgi apparatus are functionally interrelated in the process of protein synthesis. Most of the newly synthesized proteins are transported from the endoplasmic reticulum to the Golgi apparatus for further modifications before the proteins are sorted to lysosomes, plasma membrane, secretory vesicles, etc (Darnell et al., 1990). Plasma membrane, endosomes and lysosomes are interrelated in the process of endocytosis. Materials taken up by endocytosis are transported via plasma membrane to endosomes and then to lysosomes. Some membrane proteins have been shown to cycle between plasma membrane, endosomes and lysosomes (Lippincott-Schwartz and Fambrough, 1986 and 1987; Muller et al., 1980; Taylett and Thilo, 1986). These interorganelle relations, or reciprocal organelle dynamics, have been extensively studied and have become common knowledge for cell biologists.

In contrast, little is known about the organelle relationship within the same organelle population. For example, for multi-copy organelles, it is not known if there is any communication between different copies of the organelle; or, for single copy organelles, it is not known if resident membrane proteins are mobile within the membrane delimited area. My thesis research was designed to answer these ques-

tions with respect to resident membrane proteins. The lysosome was chosen as an example for my study of the intraorganelle dynamics (chapter 2 and 3). My study was then extended to the comparative behavior of the pre-lysosomal compartment (PLC) and the lysosome in fused cells (chapter 4). The mobility of Golgi membrane proteins, both resident and transient Golgi membrane proteins, was also investigated (chapter 5).

1.2. Dynamics within the lysosomal population: lysosomal content and membrane intermixing in fused cells

The first description of lysosomal phenomenon can be dated as early as 1883 when Metchnikoff studied phagocytosis. But the lysosome was not named and biochemically characterized until the early 1950's when Christian de Duve, a Nobel laureate in 1974 for his work on lysosomes, and his colleagues tried to localize the glucose-6-phosphatase which proved to be a cytosolic enzyme (Bainton, 1981; de Duve, 1975). The characterization was made possible by the combination of the two techniques: cell fractionation using differential centrifugation, and morphological localization of acid hydrolase distribution using electron microscopy. The characteristics of the lysosome have been extensively studied later by many other researchers, spearheaded by Novikoff, Cohn, Hers, etc (e. g. Bainton, 1981; Novikoff, et al., 1967; Cohn, et al., 1963; Hers and ven Hoff, 1973). These studies have given us a general picture of what the lysosome is about. Briefly, lysosomes, by definition, are membrane bounded vesicles which are acid hydrolase enriched. In animal cells, there are about 300 copies of lysosomes per cell, distributed throughout the cytoplasm (Alberts et al, 1983). Under electron microscopy, they appear as dense bodies, varying in size (from about 0.2 μm to 2 μm in diameter). In plant or yeast cells, lysosomes exist as

vacuoles with only a few copies per cell. The interior of the lysosome is acidic, with a pH of 4.5 to 5.0. The main function of the lysosome, as indicated by its name, "lytic particle", is to hydrolyze macromolecules which are taken up by endocytosis or those transferred to the lysosome from the cytosol by a recognition system. The lysosome also provides nutrition for the cell. During the process of hydrolyzing the macromolecules, the degraded products diffuse from lysosomes and are used as metabolic substrates by the cell. These functions are fulfilled by the existance of over 50 different kinds of lysosomal acid hydrolases, which have optimal enzyme activity at acid pH. The lack of hydrolase(s) would lead to the accumulation of macromolecules, resulting in the enlargement of lysosomes and malfunction of lysosomes. In some instances, enayme deficiancies cause of a inherited lethal diseases. I-cell disease and Tay Sachs are two examples (Glew, 1985). The lysosomes also play an important role in the immune system. Firstly, in phagocytes (mainly polymorphonuclear leukocytes and macrophages in vertebrates), there are specialized lysosomes which are full of peroxidase and are responsible for killing and digesting the bacteria and other foreign particles engulfed by the cells (Adams and Hanna, 1984). Secondly, the lysosome may well be the place that, in B lymphocytes and phagocytes, foreign molecules are degraded and processed (Gupta, 1986) into foreign antigens. These antigens are then bound to major histocompatibility complex (MHC) and returned to the B lymphocyte or phagocyte cell surface. This will stimulate the proliferation of T helper cells and eventually stimulate the production of antibodies (Holtzman. 1989).

The biogenesis of lysosomes is an important topic in cell biology. Lysosomal contents, mainly the acid hydrolases, are synthesized in the endoplasmic reticulum, where they are partially glycosylated. The hydrolases are then transported to the Golgi apparatus to have further modifications. One modification is that the end of

some carbohydrate side chains become mannose-6-phosphate positive, a unique feature for the acid hydrolases. Then the hydrolases are delivered from the Golgi apparatus to lysosomes by a receptor mediated pathway (von Figura and Hasilik, 1986). In mammalian cells, the receptor is a mannose 6-phosphate receptor (MPR) which binds specifically to the glycoprotein exhibiting mannose-6-phosphate at the end of the carbohydrate side chain. Two kinds of MPRs have been found so far in mammalian cells. One is a 215-kDa cation-independent MPR (Golberg et al., 1983; Sahagian et al, 1981; Steiner and Rome, 1982) and the other is a 46-kDa cation-dependent MPR (Hoflack and Kornfeld, 1985 a, b; Dahms et al, 1989). The 215-kDa cation-independent MPR has several repeat amino acid sequences which are homologous to the 46-kDa cation-dependent MPR, indicating that these two MPR are genetically related (Dahms, et al., 1987). Lysosomal membrane proteins, on the other hand, are delivered to lysosomes by a MPR-independent pathway, despite the facts that they also are synthesized in the endoplamic reticulum and transported to the Golgi apparatus (Barriocanal et al., 1986; Braun et al., 1989; D'Souza and August, 1986; Green et al., 1987; Lewis et al., 1985). Lysosomal acid phosphatase, which is transported to lysosomes as a transmembrane protein, was shown to be transported from the Golgi apparatus to the cell surface, from where the acid phosphatase molecules were rapidly internalized through the endosomes and then transported to lysosomes (Braun et al., 1989).

There are several reasons that lysosomes have been taken as an example in my study of intraorganelle dynamics. First of all, some recent studies have indicated that the lysosome might be an organelle population which is more dynamic than previously thought. Functionally, lysosomes are not the dead end of the endocytosis pathway. With the use of a lysosomal membrane protein-specific antibody, a 100-kDa lysos-

omal membrane protein has been found to be transported from lysosomes to endosomes and to the plasma membrane (Lippincott-Schwartz and Fambrough, 1987). Morphologically, the lysosomes are dynamic structures and their distributions, sizes and shapes are not fixed (Holtzman, 1989). Lysosomes used to be described as perinuclear vesicular bodies (Bainton, 1981). Although this description is correct most of the time, it has led to a misunderstanding that lysosomes are not flexible and are isolated from each other. In fact, that is not the case. When the pH of the culture medium changes from 7.4 to 6.5, lysosomes clustered around the nuclei move outward to the very edge of the cell. At the same time lysosomes become bigger and more rounded (Heuser, 1989). Lysosomes became tubular with phorbol myristate acetate (PMA) treatment (Swanson et al., 1987). Tubular lysosomes are also seen under the normal culture conditions in live mounted cells whose lysosomes are labeled with fluorescent dye (Ferris et al., 1987). Thin, tubular lysosomes can be easily destroyed by the fixation procedures usually used during the sample preparation for electron or light microscopy, which may be the reason that tubular lysosomes were not seen as easily as vesicle lysosomes. Individual lysosomes have also been shown to move rapidly in cytoplasm along the microtubule tracks (Matteoni and Kreis, 1987).

In previous experiments, our laboratory has shown that lysosomal contents are rapidly exchanged in fused homologous CHO cells (Ferris et al., 1987). In these experiments, one group of CHO cells were labeled with one fluorescent dye and the other group of CHO cell were labeled with another fluorescent dye. When these two groups of cell were fused together, lysosomes were found to rapidly become positive for both color fluorescent dyes. These results indicate that lysosomes may well be an dynamic organelle population, i. e., different copies of lysosomes may communicate

with each other. There are also some technical reasons that the lysosome was chosen as an example in this study. One is that lysosomal content can be easily labeled with many different markers through the endocytosis pathway; the other is that different kinds of lysosomal-specific monoclonal antibodies were available. These made the assay for lysosomal content and membrane distributions possible.

In my work I have used cell fusion to bring together two groups of lysosome into the same cytoplasm. These two groups of lysosomes could be made different in their content by labeling two groups of cells with different endocytosis markers, or different in lysosomal membrane proteins by using different species of cell lines. The distributions of the lysosomal contents were then analyzed by fluorescent light microscopy and enzyme-substrate pairing biochemical assay. The distributions of lysosomal membrane proteins were analyzed by immunofluorescent labeling and immunogold electron microscopy. In chapter 1 and 2, lysosomal contents and membrane proteins are shown to be rapidly intermixed in fused cells. The results indicate that the lysosome is a very dynamic organelle.

1.3. Comparison of pre-lysosomal compartment and lysosomes in fused cells

The pre-lysosomal compartment (PLC) is a newly defined organelle (Griffiths et al., 1988, 1990). It was first reported by Gareth Griffiths and colleagues in 1988 (Griffiths et al., 1988). When they applied the cation-independent mannose-6-phosphate receptor (MPR) specific-antibody to NRK cells, a MPR-enriched, membrane-delimited perinuclear complex was found. This MPR-enriched complex was defined as pre-lysosomal compartment (PLC) later (Griffiths, 1990). In NRK and many other cell lines, PLC exhibits a unique whirlpool-like, multi-membrane structure under electron microscopy. With immunogold staining, both the outer and

inner membrane of PLC are MPR enriched. With anti-MPR antibody immunofluorescent staining, PLC presents a Golgi-like staining pattern, usually with only one copy per cell (Griffiths et al., 1988).

Although the PLC is located adjacent to the *trans* Golgi network, it has distinct features from the *trans* Golgi network (Griffiths et al., 1988). In fact, PLC are more lysosome-related than Golgi-related. For example, the PLC and the lysosome are both lysosomal membrane protein and lysosomal enzyme positive. When cells are labeled by an endocytic marker, both the PLC and the lysosome have bulk amount of the marker. The PLC and the lysosome both have acidic pH interiors. These common features of the PLC and the lysosome suggest that the PLC and the lysosome are functionally closely related to each other. It is believed that PLC is the intermediate compartment before the lysosomal contents, acid hydrolases, are delivered to lysosomes (Griffiths et al., 1988).

In the first part of my study, lysosomes have been shown to intermix their contents and membrane proteins in fused cells. This lysosomal intermixing occurred without changing the size and the overall distribution of the lysosome, i. e., lysosomes maintain their normal small size and punctate distribution in syncytia. Because the lysosome and the PLC are two interrelated organelles, my study was extended from the lysosome to the PLC. In chapter 3, the behavior of the PLC and the lysosome was compared in the fused cells. Experimental results showed that in fused cells, the initially separate PLCs congregated, forming an extended PLC complex, while lysosomes remained punctate. These results further indicate that although the PLC and the lysosome are closely related, they are two distinct organelles. The results also suggest that there may be different pathways for different organelle's self recognization and intermixing. The role of the PLC in lysosomal content and membrane

protein exchange is discussed in chapter 4.

1.4. Golgi resident and membrane protein intermixing in fused cells

The Golgi apparatus was named after Camillo Golgi who discovered the Golgi apparatus in 1898. Although the Golgi apparatus was discovered that early, it was not well accepted as a distinct organelle before the mid 1950's because it was suspected of being an artifact of the osmium/silver staining methods. After electron microscopy was applied to the study of cell biology, accompanied with the improvement of preservation and staining methods, the Golgi apparatus was shown to be a distinct organelle (Dalton and Felix, 1954 and 1956). Later on, the morphology of Golgi apparatus was carefully studied, followed by the studies of the functional aspects of the Golgi apparatus (for review, see Farquhar and Palade, 1981).

The Golgi apparatus is located adjacent to the nucleus and is interposed between the ER and the plasma membrane. Its morphology varies in different cells and with different preparation methods. Generally, it consists of a stack of membrane delimited cisternae, which usually have a flatterned, plate-like center and more dilated rims. The stack consists of *cis, medial*, and *trans* cisternae. The *cis*-cisternae often faces the nucleus and is the entrance for the proteins which are transported from the ER. The *trans*-cisternae often faces the cytoplasmic membrane. Adjacent to or continuous with the *trans*-cisternae is the *trans*-Golgi network, which is the exit place for the proteins. The Golgi cisternae are surrounded by many small vesicles at both ends. Some studies show that Golgi cisternal stacks are connected to each other in a network (Novikoff et al., 1971; Rambourg and Clermont, 1990) , and proteins may be transported between the cisternae by direct continuities. Some other studies show that protein transport between the cisternae is via small vesicles budding from one

end of the cisternae and then fusing to the end of the next cisternae (Balch et al, 1984; Braell et al., 1984; Orci et al, 1989; Rothman et al., 1984 a, b). The general consensus as summarized in textbooks (Albert et al, 1989; Darnell et al., 1990) is that transport through the Golgi apparatus is a dissociative, vesicle mediated process.

One of the important functions of the Golgi apparatus is protein modifications, mainly protein glycosylations and sulfations (Darnell et al., 1990). The other important function is protein sorting, which has been the center of attention in recent years (Rothman, 1987). The majority of the proteins synthesized in the ER are transported into the Golgi apparatus before they are sent to different places: lysosomes, secretory vesicles, plasma membrane, etc. The *trans*-Golgi network is believed to be the place where the protein sorting takes place (Griffiths and Simons, 1986). The mechanism of protein sorting, however, is not clear. For example, it is not known how the Golgi apparatus distinguishes Golgi resident from transient proteins and therefore sorts them differently.

In the last part of my study, which is presented in chapter 5, the kinetics of transient and resident Golgi membrane protein transport between initially separated Golgi apparatus units was compared in cell fusion experiments. The results showed that the resident Golgi membrane proteins are likely to be transported between Golgi apparatus via physical contact, suggesting the resident Golgi membrane proteins are mobile. On the other hand, the transient Golgi membrane proteins are transported between the Golgi apparatus via a dissociative process, implying that the vesicles may also be the pathway for the transport of transient protein transport between different Golgi cisternae. This last result is consistent with the previous conclusions of Rothman et al.(1984a,b).

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Chaper 2. Content exchange within the lysosome: A mechanism for maintaining organelle functionality?

2.1. Introduction

Numerous reports have appeared in the literature regarding heterogeneity within the vacuolar apparatus of mammalian cells and, in particular, the lysosomal compartment. We have approached the question of lysosomal heterogeneity versus homogeneity from a different standpoint. We have reasoned that, although there may well be transient heterogeneity within the lysosomal population, the maintenance of organelle functionality must require a balanced repertoire of enzymes within each lysosomal unit. Hence we have reasoned that there must be mechanisms for maintaining lysosomal homogeneity and, as a consequence, functionality. Experimentally, we have approached this question by asking if there is a rapid exchange of molecules within the mixed lysosomal population of fused mammalian cells (Fig. 2-1). In our experiments, donor and recipient cell lysosomes have been content labeled by the accumulation of endocytized solute. The distribution of markers or biochemical products resulting from organelle intermixing was scored at various times after cell fusion.

The major outcome of this work has been to show lysosomal content intermix extensively after cell fusion. This intermixing is sensitive to ATP inhibitors and

partially sensitive to cytochalasin D, an inhibitor for both microfilament and glucose uptake.

2.2. Materials and methods

Homologous and heterologous cell fusions were performed as previously described (Ferris et al., 1987). In some experiments, CHO cells fed [^{14}C]sucrose (15 μC/ml) for 18 h followed by a 2 h chase were fused with 3T3 cells which had been incubated in parallel with yeast invertase (0.5 mg/ml, grade III, Sigma Chemical Co., St. Louis). In other experiments, CHO cells were fed sucrose for 18 h followed by a 1-2 h chase to produce sucrosomes, sucrose swollen lysosomes (DeCourcy and Storrie, 1990), and fused with 3T3 cells containing organelles labeled with Texas red dextran in a parallel uptake protocol. Texas red dextran localization relative to sucrosomes in cell syncytia was determined by fluorescence microscopy. For ATP depletion, cell syncytia were incubated with 50 mM 2-deoxyglucose and 1 mM sodium cyanide. This produced >80% depletion of cellular ATP levels using the luciferin-luciferase assay. The frequency of tubular or reticular lysosomes in CHO, HeLa, NRK, and 3T3 cells was assessed in cell cultures which had been pre-fed fluorescent dextrans for 18 h.

2.3. RESULTS

Content Exchange Between Lysosomes is a Rapid Process with Minimal Time Lag

Previous results had suggested that, upon cell fusion, there might be a significant 30-45 min, temperature dependent lag in lysosomal content interchange (Ferris et al., 1987). Based on the temperature sensitivity of the lag period, we had suggested that a "priming" process might be occurring during this period (Ferris et al., 1987). The previous experiments were done chiefly by scoring the transfer of endocytized contents from donor lysosomes into recipient sucrosomes. As sucrosomes are large, dilution of lysosomal contents upon transfer into sucrosomes may have limited our ability to score early transfer events. We have reinvestigated this question using an invertase-[^{14}C]sucrose content pairing in the donor 3T3 and recipient CHO cell lysosomes. With this pairing, content intermixing results in the invertase-dependent cleavage of sucrose to glucose and fructose, which permeate cell membranes and appear rapidly in the extracellular media. As shown in Fig. 2-2, almost no lag in apparent content intermixing was detectable with this assay upon fusion of CHO and 3T3 cells. Based on this more sensitive assay, we conclude that there is no significant lag period in lysosomal content exchange upon cell fusion and that any "priming" process must indeed be rapid.

Effect of Inhibitors on Lysosome-Lysosome Exchange

In previous experiments, we have shown that lysosomal intermixing is sensitive to low temperature (Ferris el al., 1987). As shown in Fig. 2-3, lysosomal intermixing is also strongly ATP dependent, with almost complete inhibition of transfer of Texas red dextran from 3T3 lysosomes to CHO cell sucrosomes upon addition of deoxyglucose and sodium cyanide to the culture. It should be noted that sucrosomes have been shown previously to be lysosomes, i.e., lmp-positive and MPR-negative structures (DeCourcy and Storrie, 1990). We also have asked if microfilaments might be required for lysosomal content exchange. In experiments using a pairing of invertase- and [^{14}C]sucrose-positive lysosomes, we found about a 50% inhibition of lysosomal content exchange with the addition of 5 μg/ml cytochalasin D to the culture (data not shown). This inhibition may be due in part to an ATP-dependent effect. Cytochalasin D does inhibit glucose uptake by cells.

Observations on the Role of Reticular Lysosomes in Lysosome-Lysosome Exchange

A reticular nature to the vacuolar apparatus has been noted by other investigators (e.g., Swanson, Bushnell and Silverstein, 1987; Hopkins et al., 1990). Conceivably, molecular exchanges within the lysosomal population of fused cells could be the result of reticular continuities between organelles. This is a point of current research interest on which a number of initial observations can be reported. As shown previously, when CHO cells containing Texas red- and FITC-dextran marked lysosomes were fused, reticular structures were observed in live mounted cells and these were positive for both colored dextrans (Ferris et al., 1987). If these reticular structures are important in lysosome-lysosome exchanges, then the rate of

molecular exchanges should vary with the reticularity of the donor cells. To test this, a series of cell lines differing in the frequency of reticular lysosomes were fused with sucrosome-positive CHO cells. The cell lines were NRK and HeLa cells, which contain few, if any, reticular structures, CHO cells, which were intermediate, and 3T3 cells, in which reticular lysosomes were common. In this experiment, the disappearance of sucrosomes as a result of invertase exchange was scored. No consistent correlation between the extent of reticular lysosomes in the donor cell and the rate of disappearance of sucrosomes was noted; $t_{½}$'s were: NRK cell donor, 30 min; HeLa cell donor, 1 h; CHO cell donor, 25 min; and 3T3 cell donor, 20 min.

2.4. Discussion

The observations reported here and in previous work (Ferris et al., 1987) clearly suggest that within the vacuolar apparatus of mammalian cells there is a continuous molecular exchange between members of the lysosomal population. This exchange, when traced by endocytized content markers, is ATP dependent, temperature sensitive and microtubule dependent. Reticular lysosomes may play a key role in this process. However, initial data suggest no correlation between the frequency of reticular lysosomes in the donor cells and the rate of lysosomal content exchange.

Alternatively, intermixing within the lysosomal population may be due to direct, transient fusion within the organelle population. By time-lapse video microscopy of cells containing organelles labeled by long-term internalization of fluorescent dextran, transient collisions have been observed between lysosomes in the cell periphery (Deng and Storrie, unpublished observations). These collisions may lead to repeated rounds of exchange. Reticular connections between organelles are another possibility, although initial data do not support this suggestion. At present, a firm conclusion can not be reached as to which of the possible pathways is predominant for content within the lysosomal population. These observations of rapid molecular exchange within the lysosomal population do suggest that lysosomes over time should exhibit homogeneity, although at any given instant the population

may exhibit heterogeneity. Hence the balanced functionality of each organelle unit should be maintained over time.

2.5. References

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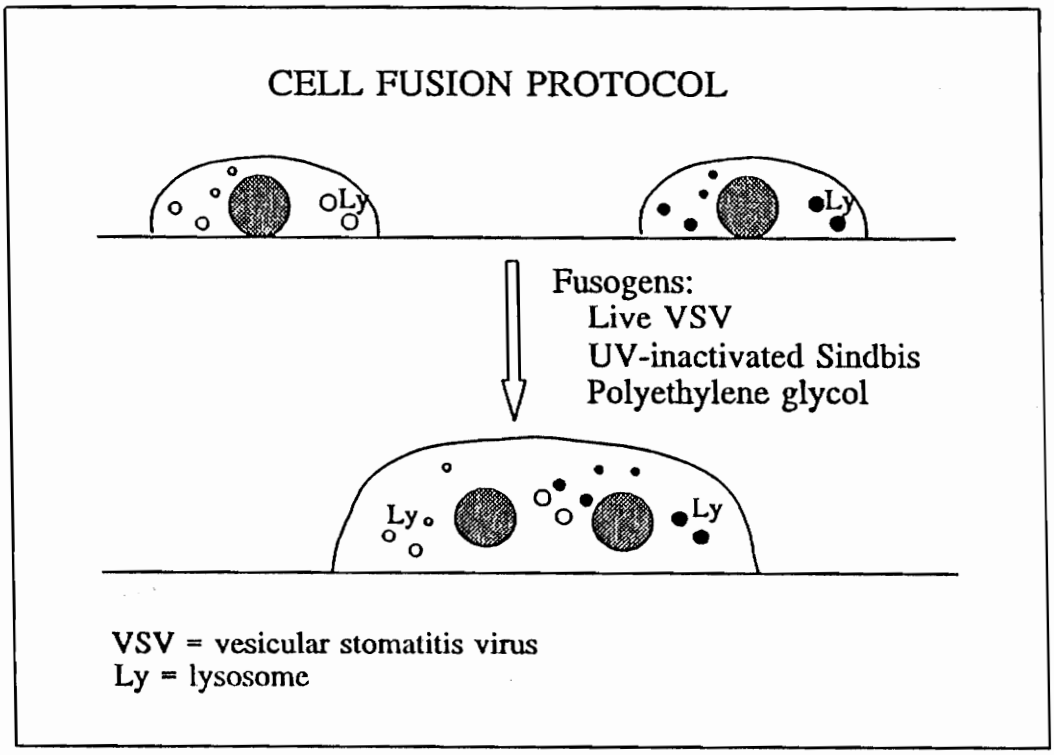

Fig. 2-1. Creation of a mixed syncytoplasm

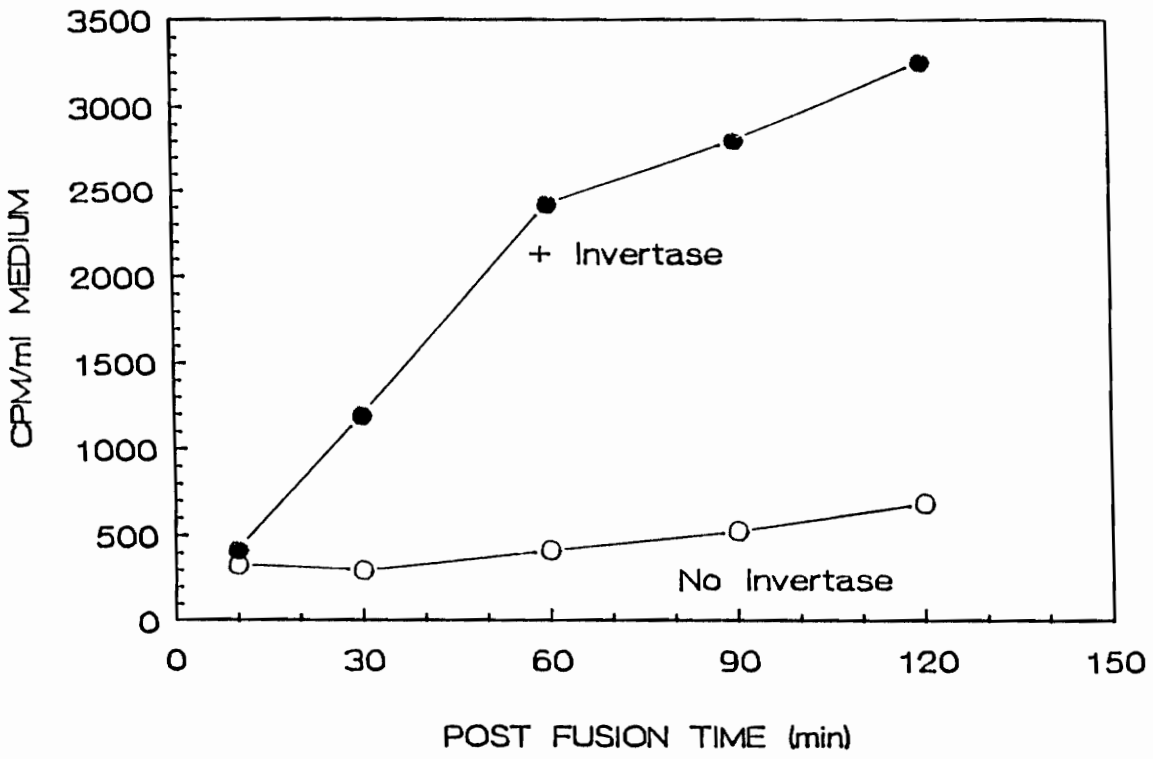

Fig. 2-2. Results of invertase-[^{14}C]sucrose assay for lysosomal content intermixing. CHO cells were incubated with 15 μC/ml [^{14}C-sucrose overnight. 3T3 cells were incubated with 0.5 mg/ml invertase overnight. Cells were incubated with marker-free medium for 2 h before cell fusion. CHO and 3T3 cells were fused together as described (Ferris et al., 1987). At different time points, post cell fusion, 5 μl of culture medium was taken out and the CPM was measured by scintillation counter.

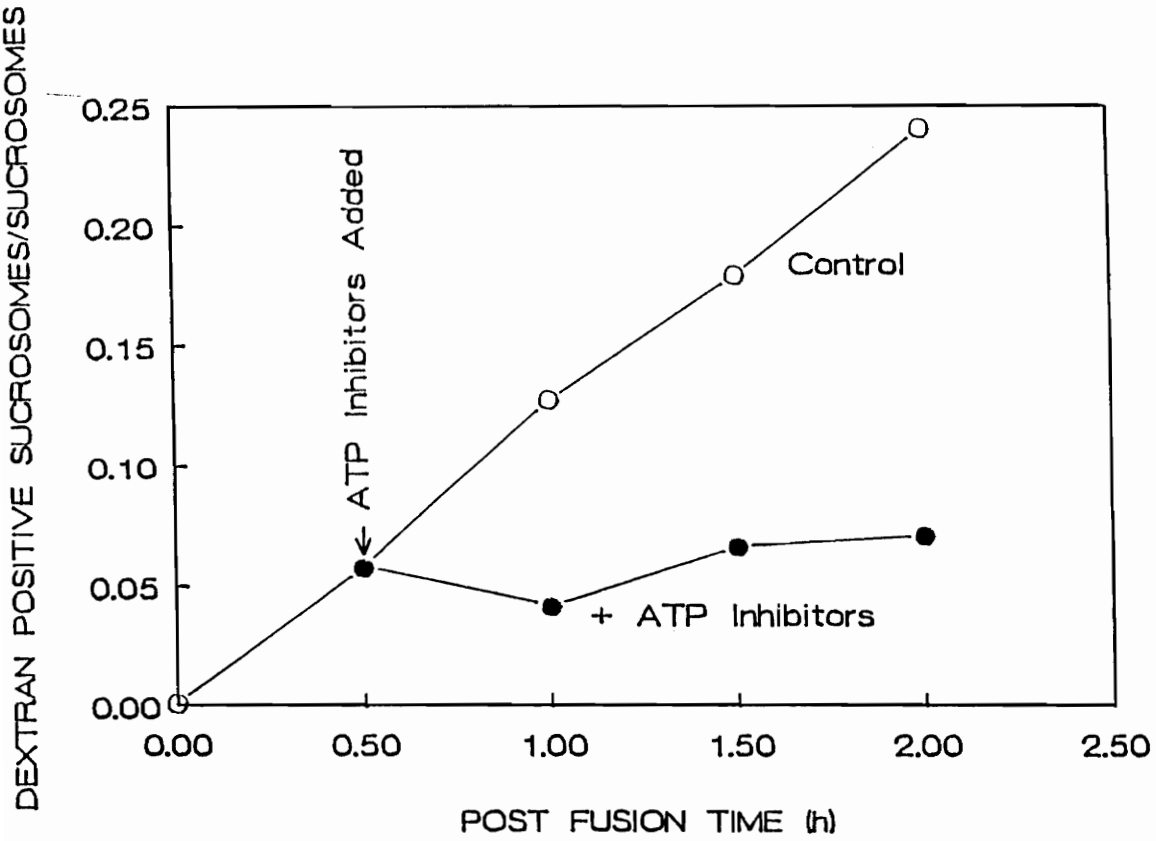

Fig. 2-3. Effect of Deoxyglucose and sodium cyanide on lysosomal content exchange. CHO cells were incubated with 0.03 M sucrose overnight to generate sucrosomes. 3T3 cells were incubated with 1 mg/ml FITC-dextran overnight. 3T3 and CHO cells were fused together as described (Ferris et al., 1987). At different time points post cell fusion, FITC-dextran positive sucrosomes were scored.

Chapter 3. Animal Cell Lysosomes Rapidly Exchange Membrane Proteins

3.1. Abstract

The lysosome has been chosen as a model to study the exchange of native membrane proteins within an organelle population. Heterologous lysosomes were brought together by vesicular stomatitis virus-mediated cell fusion. The distribution of lysosomal membrane protein was visualized by indirect immunofluorescence using species-specific monoclonal antibody. LAMP-2, a mouse lysosomal membrane protein, and HLAMP-B, a human lysosomal membrane protein, were found to transfer to Chinese hamster ovary cell sucrosomes (sucrose-swollen lysosomes). This transfer occurred in the presence of cycloheximide. The exchange of LAMP-2 and LIMP I, a rat lysosomal membrane protein, was observed between native lysosomes in a mouse (3T3)-rat (normal rat kidney) cell fusion. Extensive transfer/exchange was observed within 1.5-2 h postfusion, which is consistent with the kinetics of endocytic content exchange between lysosomes. Both membrane protein and content transfer between lysosomes were inhibited by nocodazole, a disrupter of microtubules, as was endocytic delivery to sucrose-swollen lysosomes. In the presence of nocodazole, tubular lysosomes disappeared. Both tubular lysosomes and microtubules may be important for the transfer/exchange. The interspecies cell fusion/monoclonal antibody approach developed here should be readily applicable to determine if membrane protein exchange is a property of other organelles such as

Golgi apparatus and mitochondria.

3.2. Introduction

No cellular organelle exists independently inside a cell. Like parts of a machine, organelles reciprocate and cooperate (for a general discussion see Darnell et al., 1986). Golgi apparatus, endoplasmic reticulum, and lysosomes are interrelated. Proteins synthesized in rough endoplasmic reticulum pass throuht the Golgi to the plasma membrane has been shown. Organelle resident proteins may also be transported between organelles. Mature LEP 100, a membrane protein normally resident in lysosomes, cycles between lysosomes, endosomes, and the plasma membrane (Lippincott-Schwartz and Fambourgh, 1987).

Little is known about native protein transport/exchange within an organelle population. Exchange of newly synthesized vesicular stomatitis virus (VSV) G protein, a foreign molecule, from one stack of Golgi cisternae to another has been observed in cell fusion experiments(Rothman et al., 1984). Recently, experiments done in this laboratory (Ferris et al., 1987) have indicated rapid and extensive exchange of endocytized contents between lysosomes in intraspecies cell fusion of Chinese hamster ovary (CHO) cells with CHO cells. These exchanges of foreign molecules within an organelle population raise the possibility that exchange of native organelle resident proteins may occur.

We have used the lysosome as a model to study the exchange of native molecules within an organelle population. By cell fusion, we brought together, in the same cytoplasm, lysosomes from different animal species. The distribution of species-specific membrane protein antigens between lysosomes was then detected by immunofluorescence using monoclonal antibodies. After interspecies cell fusion, animal cell lysosomes were found to rapidly exchange native membrane proteins and contents.

3.3. Materials and methods

Cell Culture. CHO-K1 (hamster) were cultured in Ham's F12 medium. 3T3 (mouse), HeLa (human), and normal rat kidney (NRK) cells were cultured in Dulbecco's modified Eagle's medium. Both media were supplemented with 10% heat-inactivated fetal bovine serum. All cells were routinely grown in 60-mm plastic tissue culture dishes at 37°C. Cells were harvested by 0.05% trypsin treatment. For cell fusion experiments , 2 X 10^5 cells were plated on 22-mm^2 glass coverslips. For viral infection, the serum supplement was lowered to 2%, and penicillin (50 units/ml) and streptomycin (50 μg/ml) were added to the medium.

Viral Stocks. Concentrated VSV stocks were prepared as described (Ferris et al., 1987). Dilutions of the VSV stocks for the cell fusion were determined empirically. If the VSV concentration was too high, the syncytia detached from the coverslips. If the concentration was too low, the extent of cell fusion was low. Different dilutions of VSV were used for each pair of cell fusions.

Lysosomal Content Labeling Conditions. All the content markers were incubated with cells in complete culture media for 18-20 h. The concentrations used were sucrose, 0.03 M; yeast invertase (grade 3; Sigma), 1 mg/ml; fluorescein isothiocyanate (FITC) dextran (molecular mass = 72 kDa; Sigma), 1 mg/ml.

Cell Fusion. Eighteen to 20 hour after plating, cells grown on 22-mm^2 coverslips were infected with diluted VSV stock at 37°C. Coverslips were rocked gently over 10 min. One hour after infection, free virus was removed by rinsing the cells twice with medium, fresh medium was added, and the cells were cultured at 37°C for 4 h to give maximum VSV G protein expression at the cell surface. Donor cells (2 X 10^5) were added to the recipient coverslips and cultured in marker-free medium for 2 h at 37°C in order to chase markers from endosomes into lysosomes. At 4 h postinfec-

tion, growth medium was removed, and cells were rinsed once and then incubated with pH 5 fusion medium (Rothman et al., 1984) for 10 min at 37°C. Cells were then rapidly rinsed with growth medium and incubated at 37°C.

Immunofluorescence. Cells were rinsed three times in Dulbeccos's phosphate-buffered saline (PBS) at 24°C and fixed in 3.7% paraformaldehyde in PBS containing 0.03 M sucrose for 10 min at 24 °C. Cells were rinsed three times with PBS and the incubated with the appropriate monoclonal antibody in 0.1% saponin for 15 min at 37°C. Coverslips were rinsed three times with PBS and then incubated with FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG (Cappel, Organon Teknika, West Chester, PA) second antibody for 30 min. In double-label experiments, FITC-conjugated goat-anti rat IgG (no cross-reactivity with rat IgG, Jackson ImmunoResearch, West Grove, PA) second antibodies were used. All antibodies were diluted in PBS containing bovine serum albumin at 1 mg/ml to block nonspecific binding. The coverslips were washed in PBS three times and then were mounted with glycerol containing *p*-phenylenediamine to reduce fading during microscopy (Plat and Michael, 1983).

Light Microscopy. Cells were photographed with a Zeiss IM-35 inverted microscope with a Zeiss Plan Neofluar X63 (numerical aperture = 1.25) oil immersion objective. Fluorescein and Texas red fluorescence was observed with selective Zeiss filter sets (Ferris et al., 1987). No fluorescein was detectable with the red filter set, and no Texas red was detectable with the fluorescein filter set.

3.4. RESULTS

Previous intraspecies cell fusion experiments have shown that lysosomes rapidly exchange content (Ferris et al., 1987). Membrane proteins alos may be exchanged between lysosomes. To test this requires bringing together in the same cytoplasm lysosomes carrying distinguishable membrane proteins. This can be done by viral-mediated cell fusion. To validate an interspecies cell fusion approach to this problem, experiments using lysosomal content markers were done. As shown in Fig. 3-1, when hamster (CHO) cells containing sucrosomes (sucrose-swollen lysosomes) were fused with mouse (3T3), rat (NRK), or human (HeLa) cells containing invertase-positive lysosomes, sucrose disappeared over a period of 1.5-2 h. This is the expected outcome for invertase transfer into all sucrosomes. The kinetics of disappearance in each species pairing was similar, but it was slower in the hamster-human cell fusion. These results indicate that content exchange occurs between lysosomes for each species pairing.

In initial membrane protein transfer experiments, we tested for the appearance of species-specific protein antigens donated by 3T3 or HeLa cells in CHO cell sucrosomes, an easily recognized lysosome model. The lysosomal membrane proteins were LAMP-2, an ~120-kD mouse glycoprotein (Chen et al., 1985; Ho and Springer, 1983), and HLAMP-B, an ~120-kD human glycoprotein (T. August, personal communication). The distribution of each of these proteins was established by immunofluorescence using species-specific monoclonal antibodies. No reaction of the monoclonal antibodies with CHO cells was observed in control experiments. Immediately after fusion of 3T3 cells with CHO cells, sucrosomes were negative for LAMP-2 staining as shown in Fig. 3-2 A and B. One to 2 h after cell fusion, LAMP-2-positive sucrosomes were frequent, and most of the total fluorescence staining

appeared to be associated with sucrosomes (Fig. 3-2 C-F). Similar immunofluorescent staining results were observed for HLAMP-B in HeLa-CHO cell fusions. As summarized in Fig. 3-3, a time lag of ~30 min was observed for the transfer of antigen to sucrosomes for HeLa-CHO cell fusion. No time lag was observed for LAMP-2 transfer in 3T3-CHO cell fusion. For both 3T3-CHO and HeLa-CHO cell fusions, the maximum frequency of antigen-positive sucrosome was observed 1.5-2 h after fusion (Fig. 3-2). In both cases, only ~50% of the sucrosomes become antigen-positive. As sucrosomes are much larger than native lysosomes, antigen dilution is likely to occur during an exchange/transfer process. Hence, the incidence of positive sucrosomes is likely to reflect the limitation of detection of antibody molecules in the staining procedure.

In these experiments, cells have been infected with VSV as a fusogen. VSV infection should result in the diversion of cellular protein synthetic machinery to the production of viral proteins. Hence, little, if any, synthesis of LAMP-2 or HLAMP-B should be occurring in the fused cells. To test for the possibility that some of the transferred antigen might be newly synthesized, experiments with cycloheximide-treated cells were done. In preliminary experiments, cycloheximide was found to inhibit >90% of [^{35}-S]methionine incorporation into proteins in a 3T3-CHO heterokaryons. The transfer of LAMP-2 to sucrosomes in the presence of cycloheximide was found to be similar to that of the control (data not shown).

To investigate if reciprocal exchange of membrane proteins occurs between native lysosomes, 3T3 and NRK cells were fused. The cell syncytia were then stained for both LIMP I (Texas Red stain), a rat lysosomal membrane glycoprotein of 35-50 k Da (Barricanal et al., 1986), and LAMP-2 (FITC stain), a mouse lysosomal membrane protein. In control experiments, no cross-reactivities were seen between the

species-specific monoclonal antibodies and the second antibodies; no fluorescein fluorescence was detected with Texas red filter set and vice versa. Five minutes after cell fusion, little, if any, overlap of the staining patterns for the two lysosomal proteins was observed (Fig. 3-4 A and B). However, 2 h after cell fusion, essentially complete correspondence between the two staining patterns was observed (Fig. 3-4 C and D). These results strongly indicated the occurrence of reciprocal exchange of membrane proteins within lysosomal populations.

To test if cytoskeletal elements (namely microtubules) have a role in the transfer of lysosomal components, 5 μM nocodazole (Swanson et al., 1987), a microtubular disruptive agent was added to cultures at various times before cell fusion. In membrane transfer experiments, the cell pairing was 3T3 cells, which are LAMP-1-positive, and sucrose-positive CHO cells. For LAMP-2 transfer, it was found that the longer the preincubation time with nocodazole, the lower the observed frequency of LAMP-2-positive sucrosomes (Table 3-1). In content transfer experiments, the 3T3 cells contained lysosomes positive for long-term internalized FITC-dextran. FITC-dextran transfer to sucrosomes, an example of content transfer, was inhibited in parallel by preincubation with nocodazole (Table 3-1). For membrane protein and content transfer, >95% inhibition was seen in cells pretreated for 45 min with nocodazole. These results suggest that cytosolic microtubules are important in lysosomal-lysosome exchange. Consistent with previous reports (Freed and Lebowitz, 1970; Herman and Albertini, 1984; Matteoni Keris, 1987; Phaire-Washington et al., 1980) that movement of endosomes and lysosomes is dependent on microtubules, nocodazole treatment inhibited the accumulation of short-term internalized FITC-dextran in sucrosomes (data not shown).

Swanson et al. (Swanson et al., 1987) have described, for macrophages tubular

lysosomal projections that appear to be stabilized by microtubules. This laboratory has shown that tubular lysosomes are common in CHO cells (Ferris et al., 1987). As tabulated in Table 3-1, FITC-positive tubular lysosomes were frequent in the control 3T3-CHO cell fusion but were rare in the nocodazole-treated fusion.

3.5. Discussion

Our experiments indicated that membrane proteins can be rapidly exchanged within an organelle population. By taking as examples three different lysosomal membrane proteins, we found in interspecies cell fusion experiments that each protein distributed through the lysosomal population over a time span of 1.5-2 h. The proteins were recognized by species-specific monoclonal antibodies. This cell fusion/antibody approach should be applicable to other organelles such as Golgi apparatus and endoplamic reticulum.

The distribution of lysosomal membrane proteins was visualized by immunofluorescence. Immunofluorescence was chosen because it provides a rapid and overall sense of the distribution of the proteins. In single-label cell fusion experiments, LAMP-2 and HLAMP-B were found to be transferred to hamster sucrosomes. Sucrosomes are a large, easily observed lysosomal population swollen by the long-term internalization of sucrose (Cohn and Ehrenreich, 1969). Sucrosomes, because of their size, rapidly permit the assignment of antigen staining to organelle membranes. In double-label cell fusion experiments, essentially a complete correspondence of punctate distributions for LAMP-2 and LIMP I was observed.

Cell fusion was mediated by VSV. VSV infection results in the expression of the viral G protein at the cell surface. At acid pH, the G protein is fusogenic. During the time course of these experiments, the cell syncytia appeared healthy. The pathway of protein glycosylation and hence membrane trafficking is normal in VSV- infected cells (Ghosh, 1980). Pinocytic vesicles fuse normally with lysosomes in VSV-infected cells (Wilcoz et al., 1983). Therefore, VSV-fused cell system should be a good model for organelle-organelle exchanges.

Treatment of cell syncytia with nocodazole inhibited both lysosomal membrane

protein and content exchange. With nocodazole treatment, tubular lysosomal structures disappeared, which is in agreement with previous work (Swanson et al., 1987). These results suggest a role for both cytoskeletal elements and tubular lysosomes in lysosome-lysosome exchange. The primary effect of nocodazole is a consequence of its binding to tubulin, the subunit protein of microtubules. However, nocodazole also produce secondary effects, such as the disruption of intermediate filaments (Goldman, 1971). Hence, the determination of the exact role of microtubules in organelle exchange will have to await further experiments.

The correlation between lysosomal membrane and content exchange and the occurrence of tubular lysosomes suggests an important role of this structure in lysosome-lysosome exchange. Swanson et al. (Swanson et al., 1987) have speculated that lysosomes may be interconnected by tubules to form a lysosomal reticulum. Lysosome-lysosome exchange processes could occur by bulk transfer through this reticulum. Membrane proteins would freely exchange by diffusion in the lipid bilayer and soluble proteins would exchange by diffusion in the reticulum. Lysosome-lysosome exchange may also occur by fusion/fission between discrete organellar elements or by carrier-mediated processes. At least in the case of LEP-100, a chicken lysosomal membrane protein (Lippincott-Schwartz and Fambourgh, 1987), the lysosomal membrane proteins is in equilibrium with endosomes, which are a potential carrier population.

Note Added. LAMP-2 and HLAMP-B have been recently renamed mLAMP-2 and hLAMP-2, respectively.

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Table 3-1. Nocodazole inhibits the transfer of LAMP-2 and FITC-dextran from 3T3 lysosomes to CHO sucrosomes and produces a loss of tubular lysosomes

Treatment	Transfer inhibition, %		Tubular lysosomes
	LAMP-2	FITC-dextran	
None(control)	0	0	+++
Nocodazole			
0 min	61.0	59.4	-
15 min	84.0	70.7	-
30 min	96.6	87.4	-
45 min	96.3	96.5	-

Nocodazole (5 μM) was added to cell cultures for various times prior to cell fusion. All fused cells were photographed 2 h postfusion. LAMP-2 was localized by indirect immunofluorescence. 3T3 cells were cultured in the presence of FITC-dextran at 1mg/ml to produce dextran-positive lysosomes, followed by a 2-h chase period in marker-free medium. Tubular lysosomes were scored in the FITC-dextran portion of the experiment (+++, frequent; -, rare). The fixation conditions for the indirect localization of LAMP-2 by immunofluorescence do not preserve tubular lysosomes. The percentage of transfer inhibition = [1-(%LAMP-2- or FITC-dextran- positive sucrosomes with nocodazole/%LAMP-2 or FITC-dextran positive sucrosomes in control0] X 100%. (About 300 sucrosomes were scored for each experimental point.)

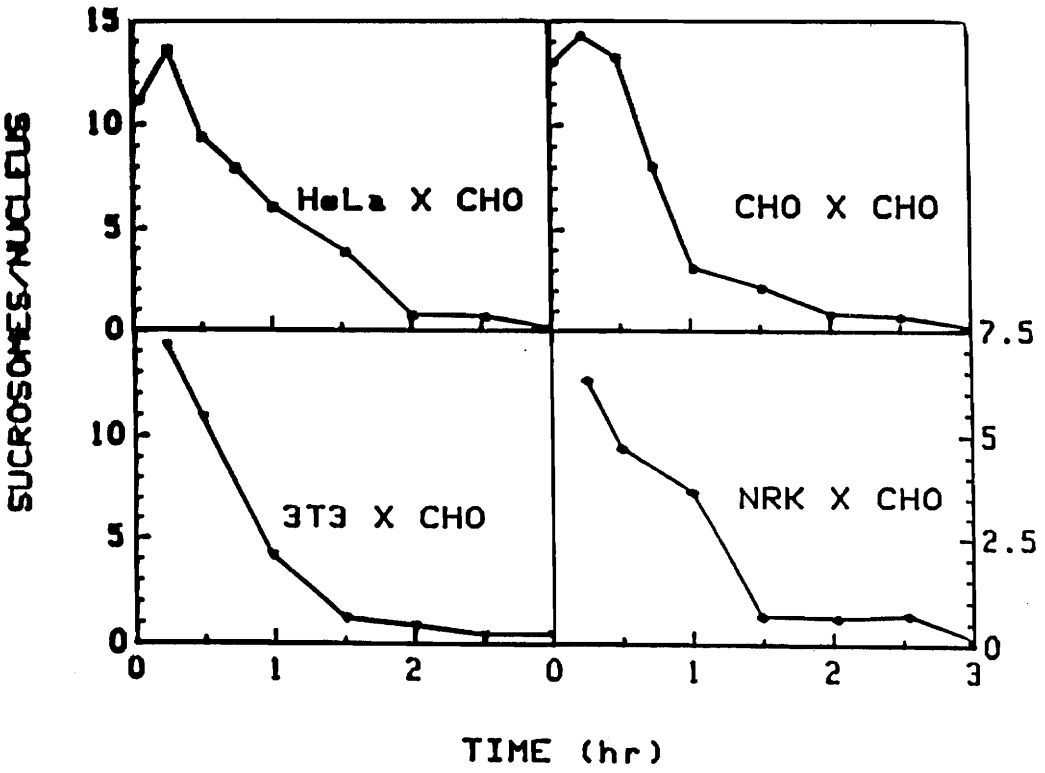

Fig. 3-1. Disappearance of sucrosomes in heterologous and homologous cell fusions. Recipient cells (CHO) were incubated in 0.03 M sucrose for 20 h. This produces large, swollen lysosomes (sucrosomes). Lysosomes of donor cells (3T3, HeLa, NRK, and CHO) were invertase-labeled by incubating the cells in invertase at 1 mg/ml for 20 h. Both donor and recipient cells were cultured in marker-free, complete culture medium for 2 h before cell fusion to clear any marker from endosomes (Storrie et al., 1984). Scoring was as described (Ferris et al., 1987).

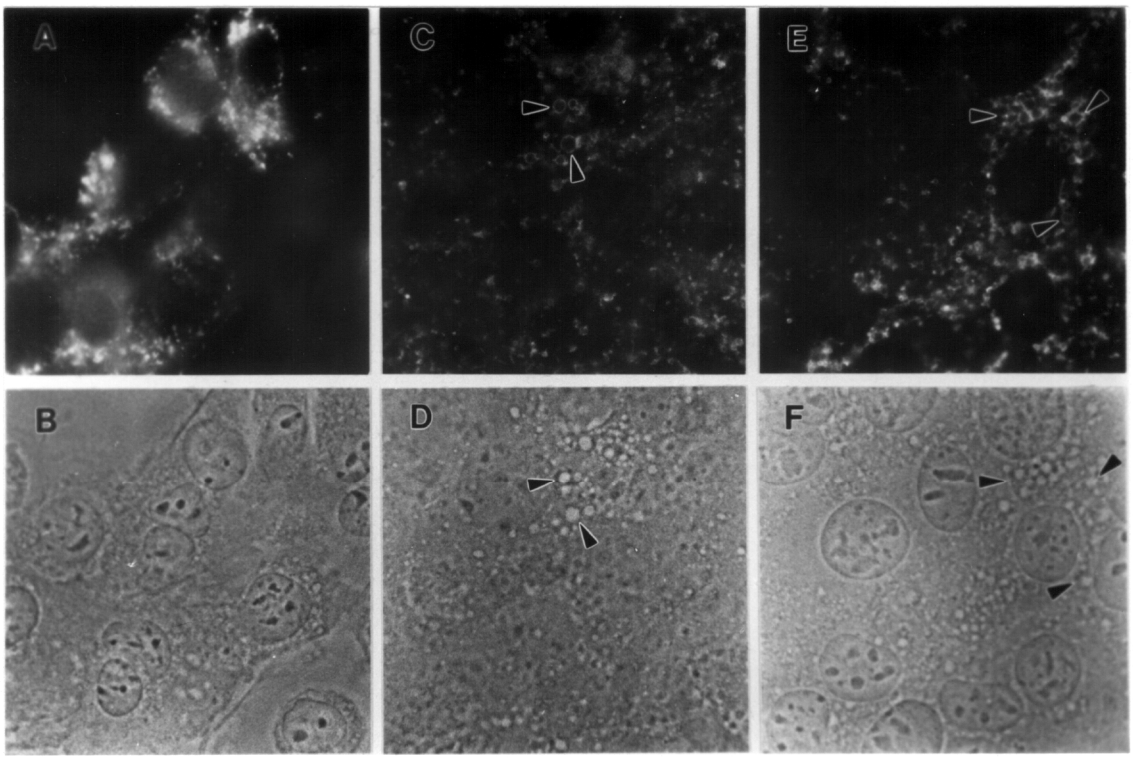

Fig. 3-2. Transfer of LAMP-2 to CHO cell sucrosomes. 3T3 cells (mouse donor cells) were LAMP-2-positive. Fused cells were fixed at different times. LAMP-2 was detected by indirect immunofluorescence using monoclonal antibody M3/84 (Chen et al., 1985; Ho and Springer, 1983) and FITC-conjugated second antibody. No LAMP-2 was detected in sucrosomes 5 min after fusion (A and B). LAMP-2 positive sucrosomes were appeared 1 h after fusion (arrowheads in C and D) and more frequent at 2 h postfusion (arrowheads in E and F). The microscope was focused under fluorescence optics. Fluorescent micrographs (A, C, and E) and phase micrographs (B, D, and F) were taken in the same focal plane (X 638).

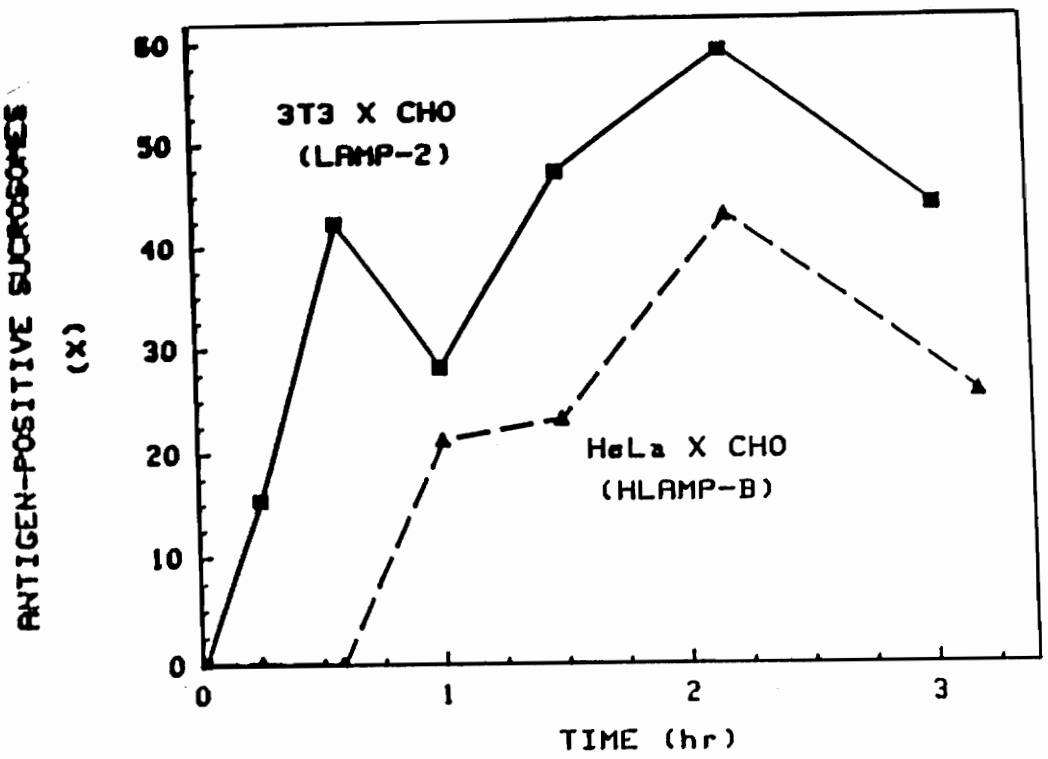

Fig. 3-3. Kinetics of appearance of LAMP-2 or HLAMP-B positive sucrosomes in heterologous cell fusions. 3T3 (LAMP-2-positive) or HeLa (HLAMP-B-positive cells were fused with sucrosome-positive CHO cells. The lysosomal membrane proteins were detected by indirect immunofluorescence by using monoclonal antibody (M3/84, LAMP-2; H4B4, HLAMP-B).

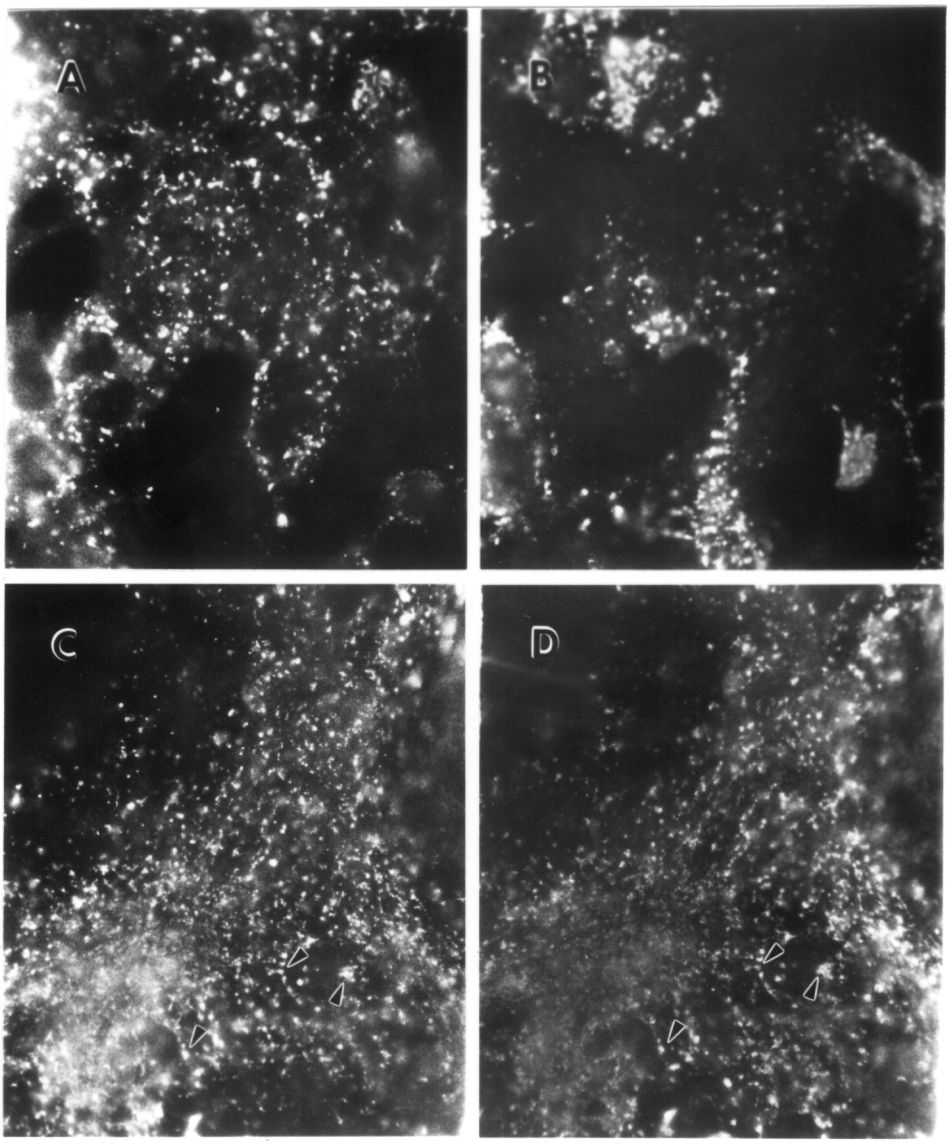

Fig. 3-4. Exchange of lysosomal membrane proteins between heterologous native lysosomes. LIMP I, a NRK lysosomal membrane protein, and LAMP-2, a 3T3 lysosomal membrane protein, were detected by species-specific monoclonal antibodies 14E12 (Barricanal et al., 1986) and M3/84, respectively. LIMP I was localized by Texas red-conjugated goat anti-mouse IgG second antibody. LAMP-2 was localized by FITC-conjugated goat anti rat IgG second antibody. Texas red (A and C) and fluorescein (B and D) fluorescence micrographs were taken at the same focus. (A and B) Five minutes after fusion, little overlap of the fluorescent punctate staining was seen. (C and D About 2 h after fusion, essentially complete overlap of the staining patterns was seen. Arrowheads point to examples. (X667)

Chapter 4. Comparative behavior of lysosomes and the pre-lysosome compartment (PLC) in in vivo cell fusion experiments

4.1. Abstract

Interspecies cell fusion was used to compare protein intermixing within the mannose 6-phosphate receptor (MPR)-enriched pre-lysosome compartment (PLC) and within the MPR-negative lysosomal compartment. Both compartments were positive for lysosomal glycoprotein (lgp) membrane markers but were morphologically distinct. In most experiments, rat-mouse cell syncytia were formed by UV-inactivated Sindbis virus mediated fusion. By immunogold electron microscopy of syncytia, extensive intermixing of species-specific lysosomal membrane proteins was observed both in lysosomes and PLC. Three h post cell fusion, multiple label immunogold studies showed that 82% of the lysosome-like structures positive for the rat lysosomal membrane protein LIMP-I were also positive for the mouse lysosomal membrane protein mLAMP-1. By immunofluorescence, LIMP-I and mLAMP-1 co-localized with a $t_{1/2}$ of 30 min after cell fusion; although the lgp-positive organelle populations had evidently interchanged their proteins, the lysosomal structures remained small, punctate bodies distributed throughout the syncytoplasm as observed in single cells. In contrast, the initially separate units of the PLC congregated with a $t_{1/2}$ of 1 h to form large, pre-lysosome complexes associated with individual nuclear clusters.

At the electron microscope level, gold markers endocytized by the rat and mouse parent cells in a 1 h uptake followed by a 16 to 20 h chase co-localized in these extended PLC complexes, as did the membrane markers mLAMP-1 and LIMP-I. The density of labeling for rat MPR in the extended PLCs was markedly decreased, consistent with membrane fusions and dilution of the antigen upon congregation of the PLC compartments from the donor cells. The extended PLC complex behaved as a late endocytic compartment, as shown by co-localization of the MPR and rhodamine dextran following a 10 min dextran uptake and a 50 min chase. These differences in behavior between lysosomes and the PLC in rat-mouse cell syncytia suggest that the pathway(s) of protein intermixing with respect to the two organelles may be different.

4.2. Introduction

Lysosomes are an acidic, membrane-bound organelle population rich in hydrolytic enzymes which are synthesized in the rough endoplasmic reticulum together with many proteins destined for other organelles. In mammals lysosomal hydrolases acquire mannose 6-phosphate residues on their oligosaccharide side chains at a post-endoplasmic reticulum site. This covalent modification enables these proteins to bind to mannose 6-phosphate receptors, a process which leads to the subsequent delivery of the hydrolases to lysosomes (for recent reviews, see Storrie, 1988; Kornfeld and Mellman, 1989). In mammalian cells, there are two different mannose 6-phosphate receptors, a ~300 kDa cation-independent receptor (CI MPR) and a 46 kDa cation-dependent receptor, both of which are integral membrane proteins (for review, see Dahms et al., 1989). Lysosomal membrane proteins also are synthesized in the rough endoplasmic reticulum, transported to Golgi apparatus and from there delivered to lysosomes by a mannose 6-phosphate independent pathway (Barriocanal et al., 1986; Braun, Waheed, and von Figura, 1989; D'Souza and August, 1986; Green et al., 1987; Lewis et al., 1985).

A compartment displaying both endosomal and lysosomal traits appears to be an intermediate structure in the transport of endocytized molecules, lysosomal hydrolases and membrane proteins to lysosomes (Geuze et al., 1988; Griffiths et al., 1988, 1990). This compartment has been variously termed a late endosome (Kornfeld and Mellman, 1989), an endolysosome (Alberts et al., 1989) or pre-

lysosome compartment (PLC, Griffiths et al., 1988). The PLC is rich in the cation-independent mannose 6-phosphate receptor and is located in the perinuclear region of the cell. By immunofluorescence with anti-MPR antibody, it exhibits a labeling pattern similar to Golgi apparatus. Immunoelectron microscopy shows that in some cell types the compartment often contains tightly packed membrane whorls (Griffiths et al., 1988). The PLC also contains typical lysosomal membrane proteins and lysosomal hydrolases, and it behaves as a late endocytic compartment that is functionally distinct from the Golgi complex (Griffiths et al., 1988, 1990). In contrast to the PLC, lysosomes are MPR-negative, are typically found throughout the cytoplasm in many copies per cell, and appear as small, round, dense structures in electron micrographs (Geuze et al., 1988; Griffiths et al., 1988). The density differences between the PLC and lysosomes enable them to be readily separated in Percoll gradients (Griffiths et al., 1990).

In previous studies, we have shown that following cell-cell fusion donor and recipient cell lysosomes appeared to readily exchange their contents and membrane proteins (Ferris et al., 1987; Deng and Storrie, 1988). This conclusion was based on an enzyme-substrate pairing assay, on co-localization of differentially labeled fluorescent dextrans internalized by the donor and recipient cell organelles, and on immunofluorescence localization of antibodies to two distinguishable sets of lysosomal membrane proteins. In those studies, the lysosomes were defined as dense organelles in a Percoll gradient which also may

be visualized in intact cells as lgp-positive structures that accumulated soluble endocytic tracers after a 16-18 h uptake followed by a 2 h chase. Recent ultrastructural data indicate that lgp molecules are present in similar concentrations in the PLC and the lysosomal compartment; further, even after >24 h incubations, internalized particulate markers such as BSA gold distribute between the PLC and lysosomes (Geuze et al., 1988; Griffiths et al., 1989, 1990). These ultrastructural observations raise the likelihood that our previous evidence for extensive lysosomal membrane protein and content intermixing in fused cells (Ferris et al., 1987; Deng and Storrie, 1988) was due to molecular exchange in both the lysosomal compartment and the PLC.

In the present work, we have compared protein intermixing within the MPR-negative lysosomal compartment and within the MPR-enriched PLC following interspecies cell fusions. The data from immunofluorescent experiments and immunogold staining of cryosections indicated that protein intermixing occurred within both organelle compartments. For the PLC, protein intermixing was accompanied by organelle congregation. For lysosomes, a disperse punctate organelle distribution was maintained. These results raise the possibility that protein intermixing within each of these two organelle populations may occur by different pathways.

4.3. Materials and methods

Cell Culture: Substratum attached NRK cells and mouse NIH 3T3 cells were cultured in DMEM containing 10% fetal bovine serum. Mouse P338 cells were cultured in MEM supplemented with 10% fetal bovine serum. Cells were routinely grown in 60 mm Falcon tissue culture dishes. Non-essential amino acids, glutamine, penicillin and streptomycin were added to the culture media.

Internalization of Endocytic Markers:

(1) *BSA gold uptake*: Cells were fed BSA gold about 2-3 h post cell plating. The BSA gold was prepared as previously described (Slot and Geuze, 1985). NRK cells were fed 5 nm BSA gold particles (stock solution O.D. = 14.0) diluted 1:25 in complete culture medium for 1 h at 37°C. 3T3 and P338 cells were fed 16 nm BSA gold particles (stock solution O.D. = 12.2) diluted 1:10 in complete medium for 1 h at 37°C. Cultures were washed seven times with cold PBS buffer. Cells were then cultured in marker-free, complete medium for 16-20 h to chase the BSA gold from early endosomes into late endosomes (PLC) and lysosomes.

(2) *HRP uptake*: Unfused NRK cells or NRK-3T3 syncytia 3 h post cell fusion were incubated with 10 mg/ml HRP (type VI, Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C in DMEM. Cells were washed three times with warm PBS and the cultures incubated an additional 15 min in complete culture medium. This procedure labels chiefly pre-lysosome compartment (PLC)/late endosomes (Gruenberg, Griffiths and Howell, 1989; Griffiths et al., 1990; Ludwig, Griffiths and Hoflack, submitted; see also, Fig. 4-8).

(3) *Cationized ferritin*: Unfused NRK cells or NRK-3T3 syncytia 3 h post cell fusion were incubated with 100 μg/ml cationized ferritin for 10 min at 4°C in DMEM. Cells were quickly warmed to 37°C and rinsed 3 times with warm PBS. The cultures were then chased for 15 min at 37°C in ferritin-free, complete culture medium.

(4) *Rhodamine dextran*: NRK-3T3 syncytia 3 h post cell fusion were incubated for 10 min with 5 mg/ml rhodamine conjugated fixable dextran (Molecular Probes, Eugene, OR) at 37°C in DMEM. Cells were washed three times with warm PBS and the cultures incubated an additional 50 min in complete culture medium. This procedure labels chiefly pre-lysosome compartment (PLC).

Cell Fusion:

(1) *Virus mediated cell fusion*: 7 x 10^5 NRK cells were seeded on 60 mm tissue culture dishes. For immunofluorescent staining experiments, 12 mm coverslips were included in dishes at the time of cell seeding. This cell number gives 70-80% cell confluency after the cells attach and spread. Sixteen to 20 h post seeding 1 x 10^6 3T3 cells were added to NRK cultures. The 3T3 cells were allowed 2-3 h to attach and spread before adding UV-inactivated Sindbis virus as fusogen, as previously described (Ho et al., 1990). For UV-inactivation the virus was placed 50 cm from a 40 W UV-light for 25 min. For the fusion of P338 cells with NRK cells, 1 x 10^6 P338 cells were added to the NRK culture.

Cycloheximide at a concentration of 10 μg/ml was added upon cell fusion in all experiments in which cell syncytia were not subsequently pulsed with an

endocytic tracer. In some experiments, cells were pretreated with 1 μM nocodazole for 1 h prior to cell fusion. Following fusion the cell syncytia were incubated in the presence of 1 μM nocodazole. Nocodazole at concentrations as high as 10 μM has no effect on cell fusion (Ho et al., 1990).

(2) *Polyethylene glycol (PEG) mediated fusion*: Cells were cultured as above. The culture medium was removed and PEG 1500 (Boehringer Mannheim, Mannheim, FRG) diluted 1:1 in 37°C DMEM was added for 1 min. The PEG was then progressively diluted with warm DMEM per manufacturer's directions. After 8 min exposure to PEG at 37°C, the diluted PEG was replaced with complete 37°C culture medium.

Antibody Reagents:

(1) *First antibodies*: 1D4B is a rat monoclonal antibody to the mouse lysosomal membrane protein mLAMP-1 (Chen et al., 1985). It does not react with NRK cells. 38E7 is a mouse monoclonal antibody to the rat lysosomal membrane protein LIMP-I (Barriocanal et al., 1986). It does not react with 3T3 cells. The monoclonal antibodies were used as diluted hybridoma supernatants. Polyclonal rabbit anti-chicken or anti-bovine 300 kDa mannose 6-phosphate receptor antisera reacted strongly with rat NRK cells, very weakly with mouse 3T3 cells and not at all with P338 cells. The rabbit anti-bovine 300 kDa mannose 6-phosphate receptor antibody was affinity purified (Griffiths et al., 1988).

(2) *Second antibodies*: For immunogold labeling of lysosomal membrane proteins on thawed cryosections, non-crossreactive, affinity purified rabbit anti-mouse IgG

and rabbit anti-rat IgG antibodies (Jackson ImmunoResearch, West Grove, PA) were used to recognize the 1D4B and 38E7 monoclonal antibodies. These rabbit antibodies were absorbed against formaldehyde fixed, 0.2% Triton X-100 permeabilized 3T3 and NRK cells to remove non-specific binding activity to 3T3 or NRK cells.

Immunogold Labeling: Cells and cell syncytia were detached by proteinase K treatment and then fixed in 8% paraformaldehyde for 1 h at room temperature (Griffiths et al., 1984). Pelleted fixed cells were frozen in liquid nitrogen. For single labeling, cryosections were prepared and labeled with antibodies and protein A gold. For double labeling of LIMP-I and mLAMP-1, cryosections of fused NRK-3T3 cells were first incubated with the anti-LIMP-I supernatant 38E7, rinsed, incubated with rabbit anti-mouse IgG second antibody, rinsed, and then incubated with 6 nm protein A gold (Geuze et al., 1981). The cryosections were then rinsed again and incubated with protein A to saturate any free protein A binding sites. To label mLAMP-1, the cryosections were rinsed, incubated with the anti-mLAMP-1 supernatant 1D4B, and the sequence of steps described above repeated with the exception that 9 nm protein A gold was substituted to give specific labeling of mLAMP-1. Similarly for double labeling of LIMP-I and MPR, the above procedure was followed with substitution of anti-MPR antibodies and 9 nm protein A gold to give specific labeling of the CI MPR.

Immunofluorescent Staining: To triple label the NRK-3T3 cell syncytia for LIMP-I, mLAMP-1, and MPR, 3T3-NRK cells were fixed and then coincubated with

38E7 supernatant, 1D4B supernatant, and anti-MPR antibodies. Cells were then coincubated with three different colors of non-crossreactive fluorescent conjugated second antibodies (Jackson ImmunoResearch, West Grove, PA). These were: FITC conjugated, affinity purified, goat anti-mouse IgG (LIMP-I), Texas red conjugated, affinity purified, goat anti-rat IgG (mLAMP-1), and coumarin conjugated, affinity purified, donkey anti-rabbit IgG (MPR). For co-localization of rhodamine dextran and MPR, dextran fed NRK-3T3 syncytia were fixed and then stained with rabbit anti-MPR antibody and the coumarin second antibody.

Morphometry:

(1) *Quantitation of the HRP-positive structures and nucleus/cytoplasm ratio*: Unfused NRK cells or NRK-3T3 syncytia were fed with HRP (see above). Cells were fixed and the HRP reaction product developed by the diaminobenzidine reaction; the cells were then embedded in Epon (Griffiths et al., 1989). Electron micrographs of the sections were taken at 8000 X magnification in systematic fashion. Quantitation and determination of volume density of the cytoplasm and the HRP-positive structures per cell were by point counting, as described (Griffiths et al., 1989).

(2) *Quantitation of mLAMP-l and LIMP-I co-localization and the number of PLC per nucleus in cell syncytia*: NRK-3T3 syncytia were fixed and triple immunofluorescent labeled for mLAMP-l, LIMP-I and MPR. Micrographs were taken sequentially in the FITC, Texas red, and coumarin channels using a Zeiss

63x plan neofluar objective. For quantitation, micrographs printed to a final magnification of 830x were overlaid with a grid and scored for antigen co-localization and PLC number per nucleus.

(3) *Quantitation of LIMP-I and MPR labeling density in pre-lysosome compartment (PLC)*: Cryosections of unfused NRK and NRK-3T3 syncytia were double labeled with LIMP-I and MPR (see above). LIMP-I was localized with 6 nm protein A gold and MPR with 9 nm protein A gold. Micrographs of the PLC (MPR positive compartment) were taken in a systematic fashion at 34,000X magnification. After enlarging the negatives by 4.09x, the density of LIMP-I (6 nm gold) and MPR (9 nm gold) was determined by point counting as previously described (Griffiths and Hoppeler, 1986).

4.4. RESULTS

Fused Cells Have a Normal Endocytic Compartment Size and Nuclear/Cytoplasmic Ratio

Fused mammalian cells have been shown previously to have a normal metabolic activity with respect to amino acid and DNA precursor incorporation (Harris, 1970; White et al., 1981). To determine whether any gross morphological changes had occurred in our cells, we determined the volume densities of the nucleus, cytoplasm and endocytically-active organelles in thin sections of both single NRK and NRK-3T3 fused cells. In order to identify fused cells under the electron microscope, each of the parent cell populations had been incubated prior to cell fusion with either 5 nm BSA gold (NRK) or 16 nm BSA gold (3T3) for 1 h, and then chased for 18 h in marker-free media. For BSA gold, this pulse-long term chase protocol resulted in a roughly equal distribution of particles between the pre-lysosome compartment (PLC) and lysosomes (data not shown). This appeared to be a stable distribution. Following UV-inactivated Sindbis virus mediated fusion, the syncytia were incubated with horseradish peroxidase (HRP) for 30 min and then cultured in marker-free media for 15 min. At the end of this HRP pulse-chase protocol, most of the HRP should be localized to the PLC (Ludwig, Griffiths and Hoflack, submitted). As shown in Fig. 4-1, the expected localization of the HRP diaminobenzidine reaction product to the PLC was observed in cell sections doubly-positive for 5 nm and 16 nm BSA gold particles. Cell fusion had no apparent effect on the total volume density of the HRP-

positive compartment, (~1.1-1.2% of cytoplasm, Table 1). In parallel experiments, BSA gold labeled syncytia were similarly incubated with ferritin 3 h post cell fusion. The ferritin-positive compartment was 1.3% of the syncytoplasm. As shown in Table 1, cell fusion also had no effect on the nuclear/cytoplasmic ratio.

Immunogold Labeling Indicates Co-localization of Rat and Mouse Lysosomal Proteins in Both Lysosomes and Pre-Lysosome Compartment (PLC) in Fused NRK-3T3 Cells

NRK and 3T3 cells, prelabeled (1 h uptake-overnight chase) with 5 nm and 16 nm BSA gold, respectively, were fused with UV-inactivated Sindbis virus. Cycloheximide was added to prevent further protein synthesis and 3 h post fusion the syncytia were fixed and processed for immunogold labeling of cryosections. Antibodies against two distinct membrane proteins were available: a mouse monoclonal antibody 38E7 against rat LIMP-I, a 35-50 kDa lysosomal membrane protein (Barriocanal et al., 1986), and a rat monoclonal antibody 1D4B against mouse mLAMP-1, a 110 kDa lysosomal membrane protein (Chen et al., 1985). No crossreactivity of species-specific antibodies in the immunogold staining procedure was observed (results not shown). As shown in Fig. 4-2A,B, lysosomes, small membrane-bound structures with a dense interior devoid of internal membranes, were positive for both rat (6 nm gold particles, black arrowheads) and mouse (9 nm gold particles, white arrowheads) lysosomal membrane protein markers. Quantitatively, of 57 randomly selected lysosome-like, electron dense,

structures scored, 47 (82%) of the structures positive for rat LIMP-I were also positive for mouse mLAMP-1. Some of these lysosome profiles contained both internalized BSA gold particles (1 h uptake-overnight chase) donated by the rat (5 nm particles, filled stars) and mouse (16 nm particles, unfilled stars) cell parents (Fig. 4-2B,C). As the BSA gold content markers are clumped within the interior of the lysosome-like structures, even the 5 nm BSA gold content markers may be readily distinguished from the peripherally located LIMP and LAMP staining. Presumably as an outcome of the clumping of the BSA gold content markers, the distribution of content within the lysosome profiles was frequently polarized (e.g., Fig 2B). Note that in a given section, lysosome profiles may be positive for only one of the two BSA gold content markers (e.g., Fig. 4-2A). As shown in Fig. 4-2C, the putative pre-lysosome compartment (PLC), a large, relatively electron-lucent structure containing a membrane whorl in the center of the figure, was also positive for both rat and mouse lysosomal membrane proteins (black and white arrowheads). These results suggest that intermixing of species-specific membrane proteins occurs both for lysosomes and PLC in the fused cells.

Protein Intermixing in Pre-Lysosome Compartment (PLC) Was Accompanied by Congregation of the Organelle to Form Large Perinuclear Extended PLC Complexes in Fused Cells

To characterize the overall distribution of the pre-lysosome compartment (PLC) and rat and mouse lysosomes in the cell syncytia, NRK and 3T3 cells were fused, fixed at various times post fusion and immunofluorescently stained for

mLAMP-1 (red channel), LIMP-I (green channel), and the CI MPR (blue channel). Cycloheximide was included in the culture media to inhibit protein synthesis. As shown in Fig. 4-3 and Fig. 4-4, 10 min post cell fusion, mouse (Fig. 4-3A) and rat (Fig. 4-3B) lysosomes were non-overlapping in their distribution and PLC staining for rat MPR (black arrowheads, Fig. 4-3C) was much more intense than for mouse MPR (white arrowhead, Fig. 4-3C). These results were consistent with the non-crossreactivity of the mLAMP-1 and LIMP-I antibodies and the much greater reactivity of the anti-MPR antibodies with the rat MPR. Thirty min post cell fusion co-localization of mouse and rat lysosomal membrane antigens was observed (e.g., black arrowheads, Fig. 4-3D,E; see Fig. 4-4 for quantitation). By 60 min post fusion, extensive co-localization of mouse mLAMP-1 and rat LIMP-I had occurred (black arrowheads, Fig. 4-3G,H; Fig. 4-4) and the number of PLC foci per nucleus had declined significantly (Fig. 4-3I, Fig. 4-4). As shown in Fig. 4-3L,O, the decline in PLC foci (black arrowheads) corresponded, over a 2-3 h period, to formation of an extended PLC complex (see also Fig. 4-8B). At the end of 2-3 h post fusion, 97% of the structures positive for mLAMP-1 were also positive for LIMP-I (Fig. 4-3J,K,M,N and Fig. 4-4), although the intensity distribution of the antigens was not the same in all cases (compare Fig. 4-3J,K and Fig. 4-3M,N). Over the same time period, a 3- to 4-fold decrease in the ratio of discrete PLC foci per nucleus was observed (Fig. 4-4, see also Fig. 4-8B). Quantitatively, after allowance for a 10 min cell fusion period, these results indicate a 30 min half-time for the intermixing of lysosomal

membrane proteins and a 1 h half-time for formation of extended PLC complexes. Similar results were observed with polyethylene glycol fused NRK-3T3 cell syncytia (data not shown).

The apparent PLC congregation observed here is similar to that reported previously for Golgi apparatus in fused Vero cells (Ho et al., 1990). The latter fragmented after nocodazole treatment. To test if the PLC congregation shown here might be similarly affected by nocodazole, 3T3 and NRK cells pretreated with 1 μM nocodazole were fused and then incubated in the presence of drug. Under these conditions, the PLC labeling appeared punctate and dispersed at all time points (e.g., Fig. 4-5C,F) and the lysosomes appeared to be enlarged relative to controls (compare Fig. 4-5A,B,D,E with Fig. 4-5A,B,M,N). In the presence of nocodazole, co-localization of mLAMP-1 and LIMP-I was inhibited (Fig. 4-5D,E), but not completely blocked. As shown in Fig. 4-5, localized co-distribution of mLAMP-1 and LIMP-I was observed 3 h post cell fusion (black arrowheads, Fig. 4-5D,E). These results are consistent with the previously reported inhibition by 5 μM nocodazole of lysosomal membrane protein intermixing between native sized lysosomes and sucrose swollen lysosomes (Deng and Storrie, 1988).

To investigate at a higher resolution whether the formation of an extended PLC complex in the cell syncytia resulted from fusion of PLC elements from the two parent cells, immunoelectron microscopy experiments were done to assay for content intermixing within the MPR-enriched PLC compartment. The PLC may be positively identified in immunoelectron micrographs of cryosections by the

presence of MPR and endocytic tracers, and by distinct structural features, predominantly the presence of lamellar membrane sheets. In these experiments, NRK and P338 cells pre-fed 5 nm and 16 nm BSA gold (1 h uptake-overnight chase) were fused. P338 cells, unlike NRK cells, are negative for the CI MPR by our antibody labeling procedure. Hence, in a cell fusion experiment between P338 and NRK cells, content intermixing with respect to the PLC could be identified by the occurrence of MPR-positive structures (NRK contribution) containing 5 and 16 nm BSA gold contributed by the NRK and P338 parents, respectively. To test for this, syncytia containing pre-fed BSA gold were fixed 3 h post cell fusion and immunolabeled for MPR (black arrowheads, Fig. 4-6). The MPR-positive compartment (PLC) in the fused cells contained both the 5 nm and 16 nm BSA gold content markers (white and black stars), suggesting fusion of elements of the PLC from both parent cells.

Congregation of rat and mouse PLC in NRK-3T3 cell syncytia, if it is accompanied by membrane fusions, should result in dilution of immunogold labeling density for the rat markers LIMP-I and CI MPR in the extended PLC. Consistent with the weak immunofluorescent staining of mouse cells by the anti-bovine MPR antibody used, 3T3 cells exhibited essentially no immunogold staining for CI MPR (data not shown). In single NRK cells, a high density of immunogold labeling for LIMP-I (6 nm gold particles) and MPR (9 nm gold particles) was observed (Fig. 4-7A). When equal numbers of NRK and 3T3 cells were fused and then cultured in the presence of cycloheximide for 3 h prior to

fixation, the density of LIMP-I and MPR specific gold particles in the syncytia appeared to be decidedly less (Fig. 4-7B). Quantitation of LIMP-I and MPR labeling density from fused and unfused cells revealed an approximately 50% decrease in gold particle density for each of the PLC proteins, consistent with dilution of the rat proteins in the extended PLC (Table 2). This decrease was markedly greater than the 22% and 12% turnover of LIMP-I and MPR, respectively, expected in the absence of continued protein synthesis (Barriocanal et al., 1987; Sahagian and Neufeld, 1983).

To investigate if the large central extended PLC complex in NRK-3T3 cells behaved as a large, late endocytic compartment, NRK-3T3 syncytia 3 h post cell fusion were fed with rhodamine dextran for 10 min and then chased for 50 min in marker-free media. The syncytia were counterstained for MPR with a coumarin second antibody. Under these uptake conditions, the dextran (Fig. 4-8A) was concentrated in a perinuclear distribution which exhibited extensive overlap with the extended PLC complex (Fig. 4-8B). A portion of the dextran label was punctate, presumably corresponding to either residual dextran in an early endosomal compartment or to dextran which was localized in lysosomes.

4.5. Discussion

We have used the synchronized redistribution of organelle-specific proteins and cytoplasmic organelles following heterologous cell fusion as a model for investigating the comparative behavior of two members of the vacuolar apparatus of mammalian cells, the MPR-negative lysosomal compartment and the MPR-rich, pre-lysosome compartment (PLC). For both organelles, intermixing of resident proteins and contents was observed. For lysosomes at all time points, individual organelle units appeared punctate and scattered in distribution in the syncytoplasm. However, in contrast, the PLC formed extended, centrally located complexes in association with clustered nuclei. These data raise the possibility that there may be different pathways for content and protein intermixing in the two compartments. Any proposed pathway(s) must be consistent with the rapid half-time ($t_{1/2}$ = 30 min) of co-localization of the lysosomal membrane proteins, mLAMP-1 and LIMP-I, in the cell syncytia.

Cell fusion appears to be an excellent model for the study of organelle dynamics. Cells fused with UV-inactivated virus have been shown previously to incorporate normal amounts of amino acids and DNA precursors (Harris, 1970; White et al., 1981). In the present work, we have shown that cells fused with UV-inactivated Sindbis virus have a normal endocytic pathway with no alteration in the volume fraction of the endocytic compartment (PLC). Similarly, accumulation of HRP and ferritin into the PLC appeared normal and the nuclear/cytoplasmic volume ratio was unaltered. As an additional validation of

the virus-mediated fusion approach, some of the experiments were repeated using PEG-mediated fusion, similar co-localizations of LIMP-I, mLAMP-1 and MPR were observed by immunofluorescence. These results are in agreement with the previous conclusions of Ho et al. (1990) that the polykaryons behave identically to a giant cell in all respects analyzed.

The high resolution technique of immunogold electron microscopy of cryosections was used in most of these experiments, permitting the species-specific localization of both the lysosomal membrane proteins, rat LIMP-I and mouse mLAMP-1, and the MPR with respect to morphologically distinct subcellular structures. As seen by this approach, 3 h post fusion of rat and mouse cells 82% of lysosome-like structures positive for the rat lysosomal membrane protein LIMP-I were positive for the mouse lysosomal membrane protein mLAMP-1. When the PLC compartment was examined, intermixing was also observed for the rat and mouse lysosomal proteins, both normal PLC components. This was accompanied by dilution of the PLC labeling density for rat LIMP-I and rat MPR. Similarly, intermixing of endocytized BSA gold markers fed separately, 1 h uptake-over-night chase, to the rat and mouse cells prior to fusion was also seen, indicating that intermixing of contents also occurs for both compartments.

With respect to lysosomal intermixing, several pathways can be proposed. One possibility is that the PLC may be an intermediate in this process; lysosomal membrane proteins may be in rapid equilibrium between the PLC and lysosomes.

This suggestion is consistent with the equal labeling density in PLC and lysosomes of the lysosomal membrane protein lgp120 (Griffiths et al., 1990), and with the inability to chase quantitatively endocytized BSA gold from PLC to lysosomes (current work; Griffiths et al., 1988, 1990). Alternatively, intermixing within the lysosomal population may be due to lysosomal interconnections (Swanson et al., 1987), or to transient fusion within the organelle population. By time-lapse video microscopy of 3T3 cells containing organelles labeled by long-term internalization of fluorescent dextran (18 h uptake and a 2 h chase), repeated, localized collisions were observed between fluorescent structures, i.e., presumptive lysosomes, in the cell periphery (Deng and Storrie, unpublished observations). Such collisions may lead to repeated rounds of molecular exchange. Since all the markers that label lysosomes also label the PLC, we can not, at present, distinguish between these models.

For the PLC, our results suggest this organelle is capable of self-recognition which leads to coalescence and fusion. Whereas immediately after cell fusion the PLC was found in discrete foci in association with individual nuclei, 3 h post fusion the organelle formed extended complexes located centrally in the syncytoplasm in association with the clustered nuclei. In addition to containing the membrane markers from both rat and mouse parents, the PLC contained gold markers internalized by the parent cells, 1 h uptake-overnight chase, before fusion. It also behaved as a single compartment with respect to dextran uptake. This apparent coalescence presumably reflects an underlying

fusion of the PLC into a single compartment which may then lead to protein intermixing.

The organelle self-recognition property implied by our results may be a general trait of biological organelles. In a similar manner to the PLC, the Golgi apparatus (Ho et al., 1990) and a tubulo-vesicular compartment, transitional between the endoplasmic reticulum and Golgi apparatus (Schweizer et al., 1988), have also been observed to coalesce in mammalian cell syncytia (Xiao and Storrie, 1991). *In vitro*, early endosomes have been shown to fuse with each other, but not with later endocytic compartments (for review, see Gruenberg and Howell, 1989). And as shown above and earlier (Ferris et al., 1987; Deng and Storrie, 1988), protein intermixing is observed in the lysosomal population of fused mammalian cells. In all cases the apparent fusions have been organelle-specific.

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TABLE 1

THE NUCLEAR/CYTOPLASMIC RATIO AND HRP$^+$-COMPARTMENT (PLC)/CYTOSOL RATIO IN NRK-3T3 HETEROKARYONS ARE SIMILAR TO THOSE IN SINGLE NRK CELLS

	VOLUME RATIO OF PLC/CYTOSOL	VOLUME RATIO OF NUCLEUS/CYTOSOL
Single NRK Cell	0.012	0.250
NRK-3T3 Heterokaryon	0.011	0.217

The HRP-positive compartment, chiefly PLC by morphology, was labeled by a 10 min HRP uptake followed by a 50 min chase in HRP-free media at 37°C. The number of electron micrograph negatives scored was 28 for single NRK cells and 49 for NRK-3T3 heterokaryons.

TABLE 2

DECREASED DENSITY OF MPR AND LIMP-I
IN THE PLC COMPARTMENT
OF NRK-3T3 HETEROKARYONS

	MPR DENSITY (particles/μm^2)	LIMP-DENSITY (particles/μm^2)
NRK CELL	62.4	156.8
HETEROKARYON NRK-3T3	29.4	96.8
Ratio	0.47	0.62

The density of MPR and LIMP-I decrease by close to 1/2 following cell fusion. The parental cells were present in equal numbers. For single NRK cells, 34 PLC profiles were scored for labeling density and for NRK-3T3 heterokaryons 49 PLC profiles were scored.

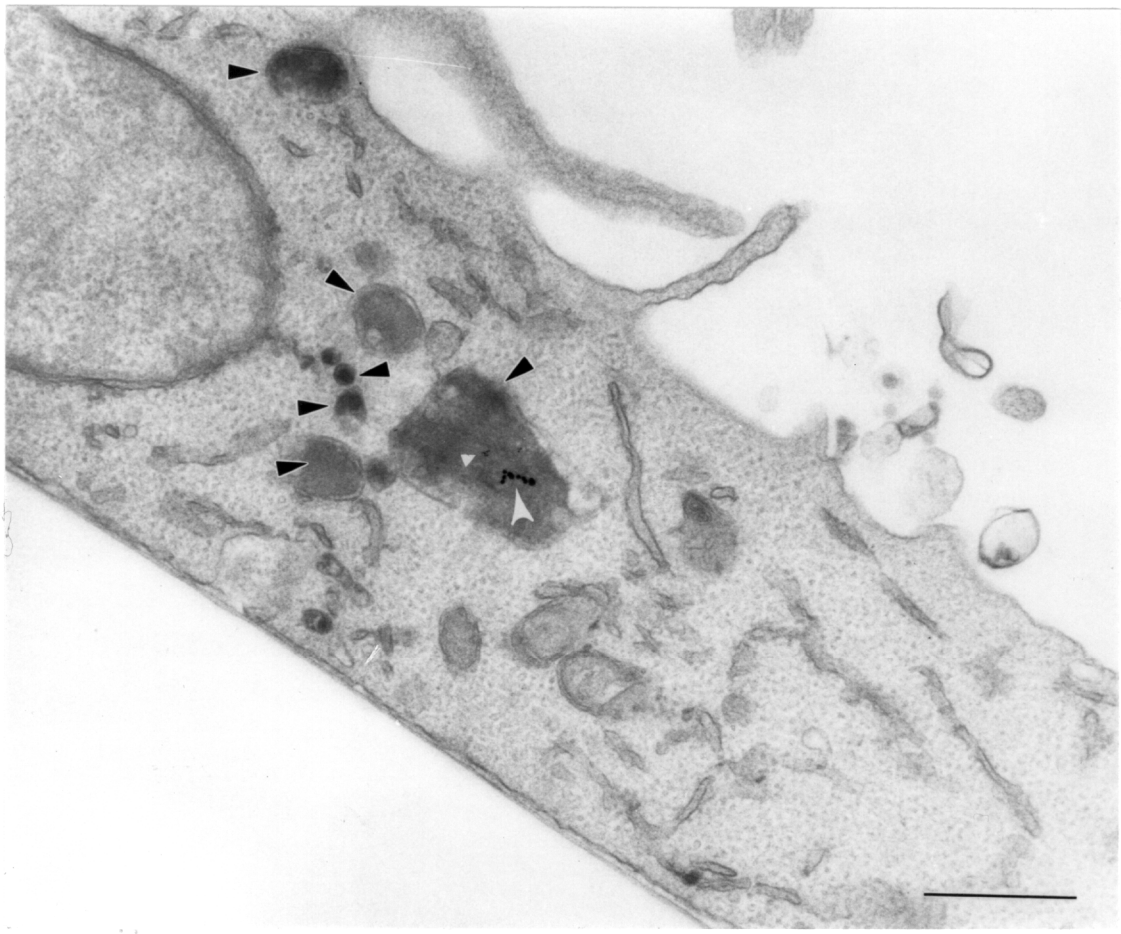


Fig. 4-1. Localization of HRP uptake by fused cells. NRK cells fed with 5 nm BSA gold and 3T3 cells fed with 16 nm BSA gold were fused. Three h post UV-inactivated virus mediated cell fusion, syncytia were fed HRP for 30 min. Following a 15 min chase in HRP-free medium, cells were fixed and processed for DAB cytochemistry. The epon embedded syncytia were sectioned and the sections were observed without counterstaining. HRP-positive vesicles are indicated by black arrowheads. The small white arrowhead points to 5 nm BSA gold particles which had been fed to NRK cells and the large white arrowhead points to 16 nm BSA gold particles which had been fed to 3T3 cells. Magnification 37,000 X, Bar = 0.5 μm.

Fig. 4-2. Immunogold labeling of NRK-3T3 fused cells showing the intermixing of LIMP-I (9 nm protein A gold, white arrowheads), mLAMP-I (6 nm protein A gold, black arrowheads), 16 nm BSA gold (white unfilled stars) fed to 3T3 cells and 5 nm BSA gold (white filled stars) fed to NRK cells. NRK and 3T3 cells were labeled with BSA gold individually for 1 h and the gold chased overnight in marker-free media. Cell syncytia were fixed 3 h post UV-inactivated virus-mediated cell fusion. Cryosections were processed for immunogold labeling. Frame A: magnification = 144,000 X, bar = 0.2 μm; Frame B: magnification = 91,000 X, bar = 0.2 μm; Frame C: magnification = 88,000 X, bar = 0.2 μm.

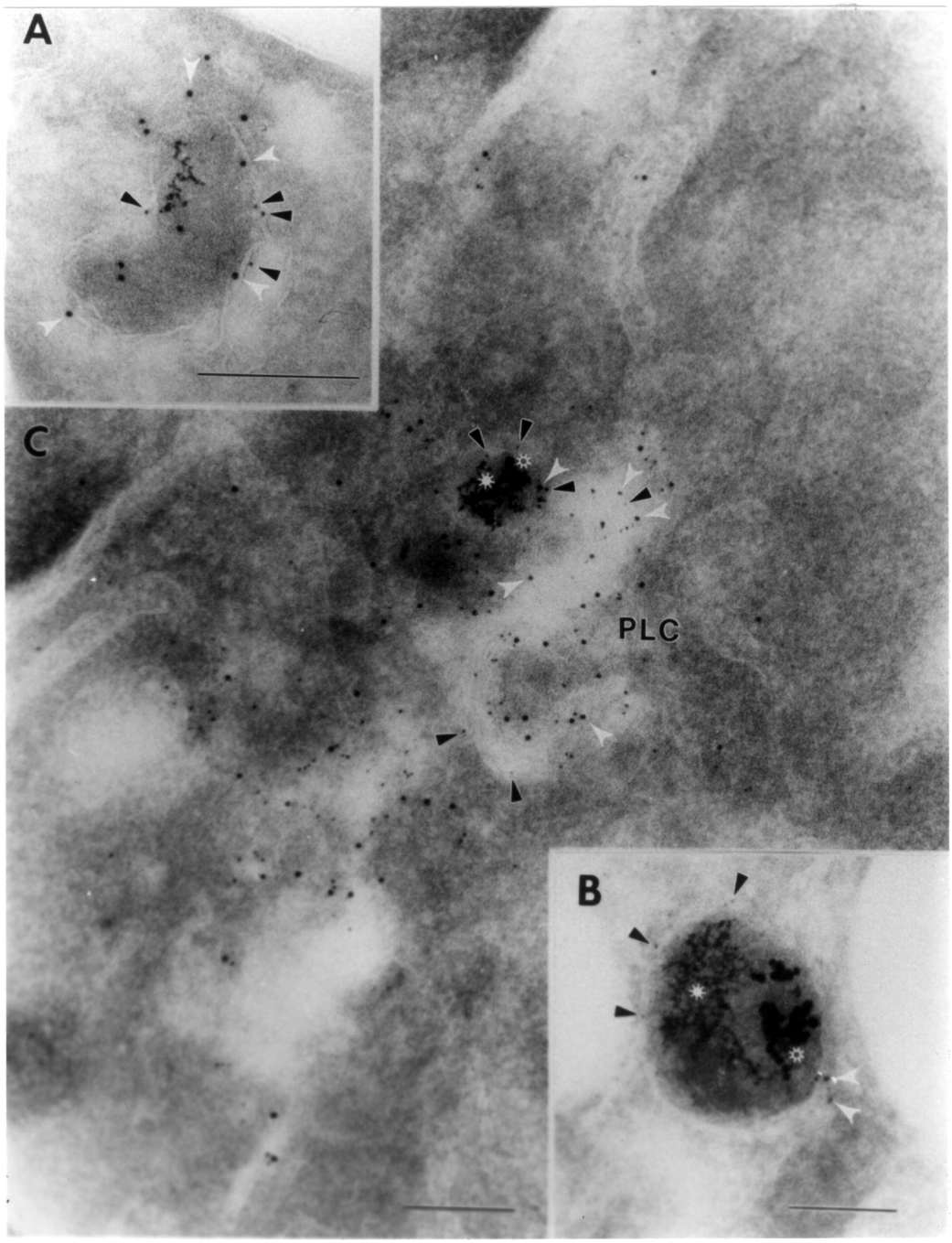
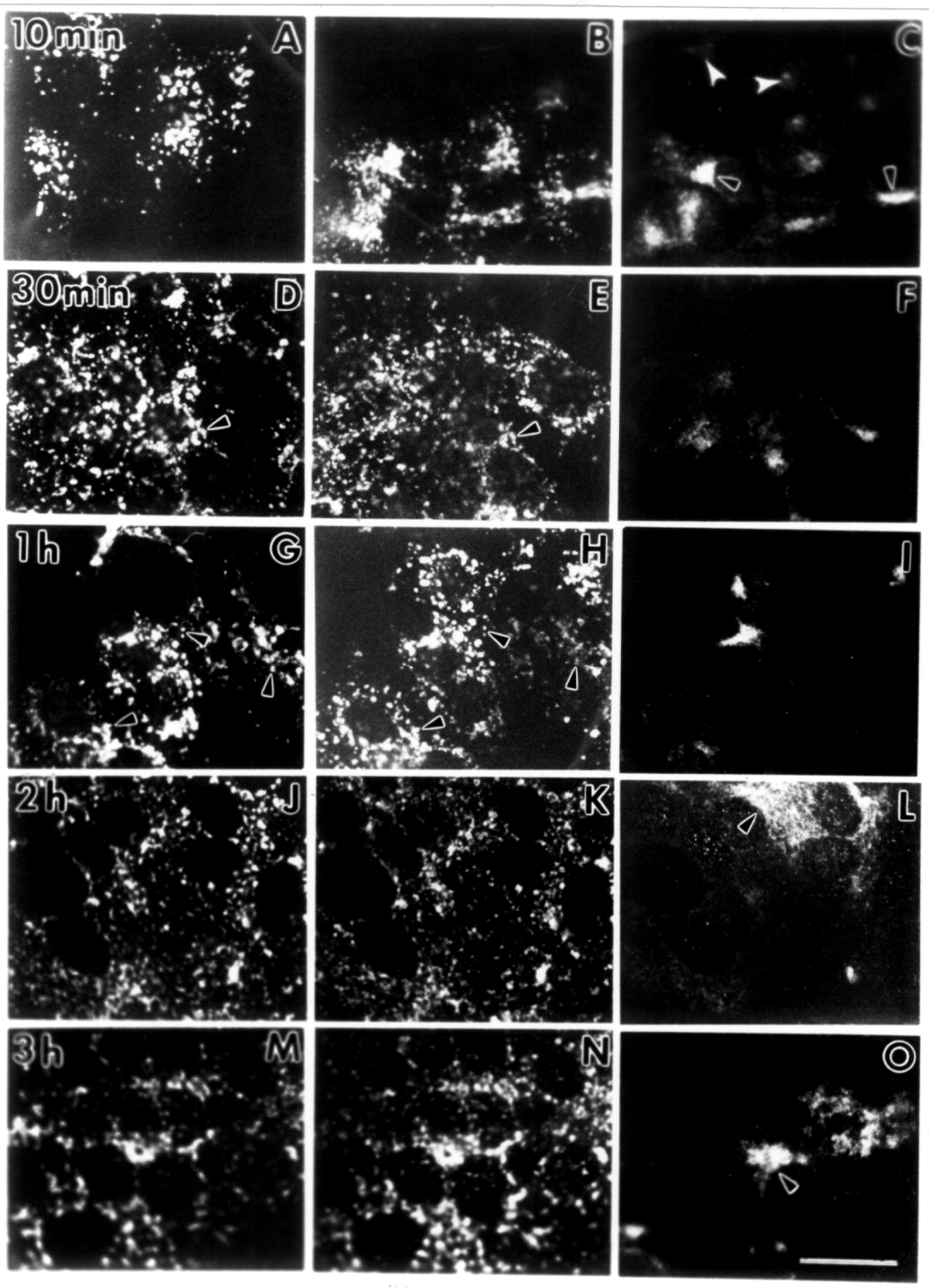

A
C
PLC
B

Fig. 4-3. Immunofluorescent localization of mLAMP-1, LIMP-I and MPR in NRK-3T3 syncytia. 3T3 and NRK cells were fused, fixed at various time points and stained for mLAMP-1 (red channel), LIMP-I (green channel) and MPR (blue channel). Micrographs in the left column (A,D,G,J,M) were taken in the red channel, those in the center column (B,E,H,K,N) were taken in the green channel, and those in the right column (C,F,I,L,O) were taken in the blue channel. For each time set, the micrographs were taken in the same focal plane. 10 min (A,B,C), 30 min (D,E,F), 1 h (G,H,I), 2 h (J,K,L), 3 h (M,N,O). White arrowheads in C point to faintly stained 3T3 PLC and black arrowheads point to NRK PLC which stain brightly. Black arrowheads in D,E,G,H point to examples of co-localization of mouse and rat lysosomal membrane proteins. Black arrowheads in L and O point to extended PLC complexes. Magnification = 614 X, bar = 30 μm.


10min
A
B
C
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1h
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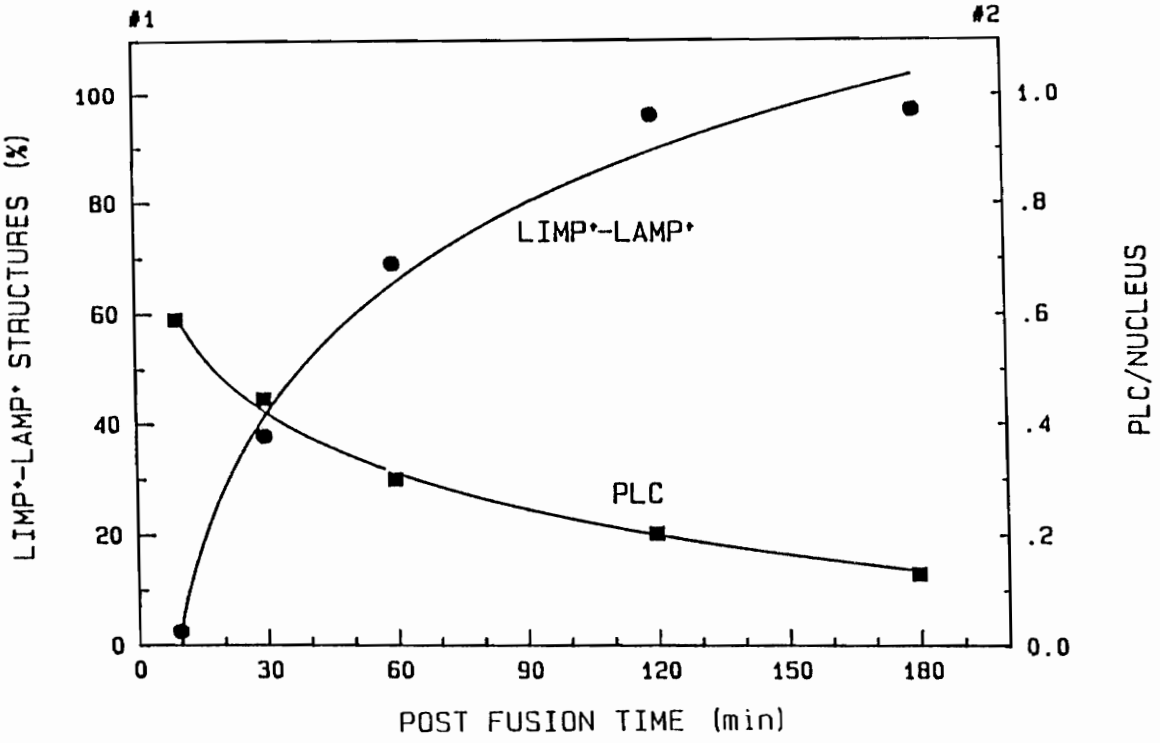


Fig. 4-4. Kinetics of LIMP-I and mLAMP-1 intermixing and of PLC coalescence in the NRK-3T3 syncytia. Micrographs from the experiment described in the legend to Fig. 4-3 were printed at a final magnification of 830 X. Quantitation of LIMP-I and mLAMP-1 co-localization was by point counting relative to a fine grid. Quantitation of the number of PLC/nucleus by scoring the number of MPR-positive fluorescent foci relative to the number of nuclei in the syncytia underestimated the contribution of 3T3 PLC; the antibody preparation recognized the 3T3 PLC marker, mouse CI MPR, very weakly. Between 10-15 cell syncytia were scored at each time point.

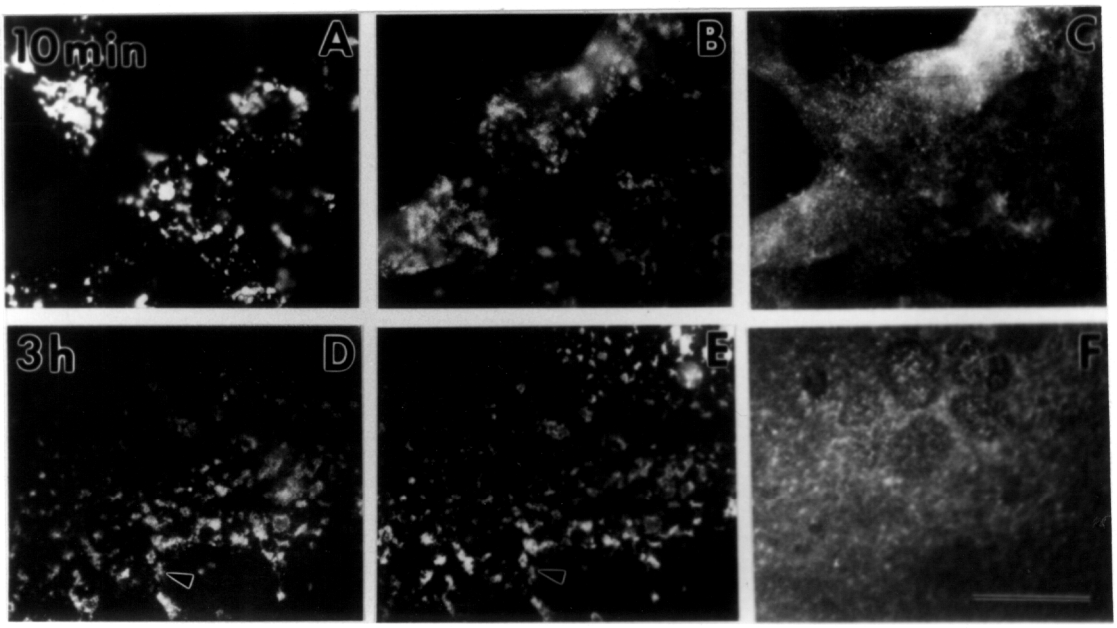


Fig. 4-5. Effect of nocodazole on the distribution of mLAMP-1, LIMP-I and MPR staining in NRK-3T3 syncytia. Cells were pretreated for 1 h with 1 μM nocodazole before fusion and then incubated in the presence of drug. The syncytia were fixed at various time points and stained for the antigens as described in the legend to Fig. 4-3. Left column (A,D): mLAMP-1 distribution, center column (B,E): LIMP-I distribution, right column (C,F): MPR distribution. For each time set, the micrographs were taken in the same focal plane. 10 min (A,B,C), 3 h (D,E,F). Black arrowheads in D,E point to examples of lysosomal membrane protein co-localization in nocodazole treated cells. In all cases, nocodazole produced dispersal of the PLC. Magnification = 614 X, bar = 30 μm.

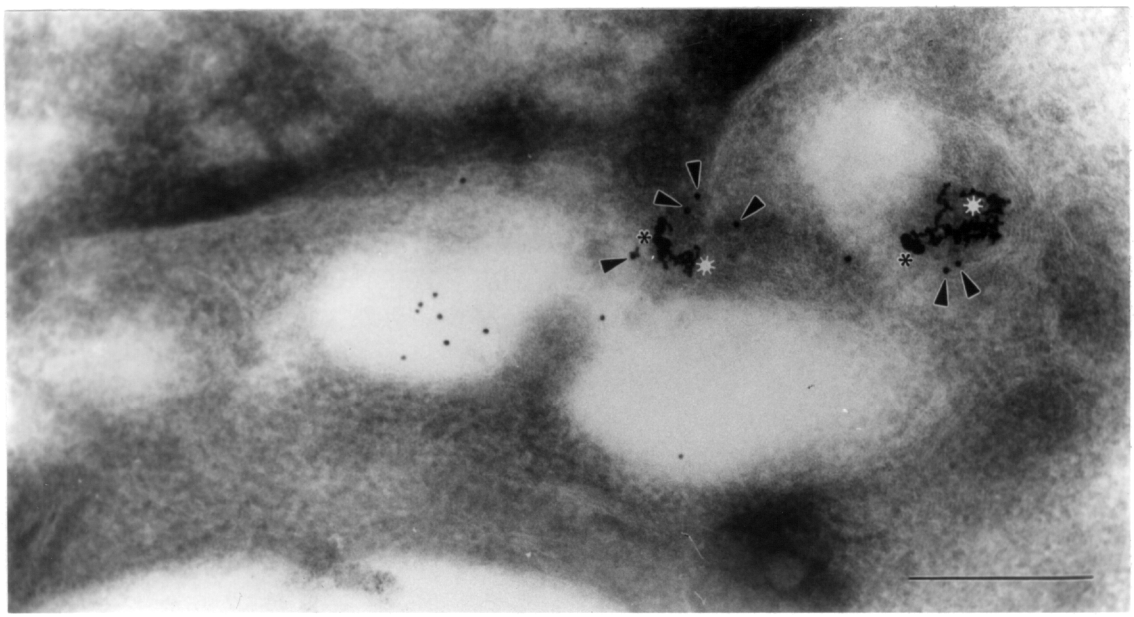


Fig. 4-6. Intermixing of BSA gold content markers in the PLC of NRK-P338 syncytia. P338 cells (CI MPR-negative) prefed 16 nm BSA gold were fused with NRK cells (MPR-positive) prefed 5 nm BSA gold. Three h post UV-inactivated virus mediated cell fusion, syncytia were fixed, cryosectioned and stained for MPR (9 nm protein A gold). Co-localization of prefed 16 nm BSA gold (P338 cells, black stars) and 5 nm BSA gold (NRK cells, white stars) was seen in MPR-positive PLC (arrowheads). Magnification = 139,000 X, bar = 0.2 μm.

Fig. 4-7. Comparative immunogold labeling of single NRK cells and NRK-3T3 cells for MPR (9 nm protein A gold labeling, white arrowheads) and LIMP-I (6 nm protein A gold labeling, black arrowheads). Frame A is a representative field showing MPR and LIMP-I labeling density in single NRK cells. Frame B is a representative field showing MPR and LIMP-I labeling density in NRK-3T3 syncytia. The syncytia were fixed 3 h post UV-inactivated virus mediated cell fusion. The 3T3 cells were prefed 16 nm BSA gold. 3T3 cells are essentially negative for reactivity with an anti-bovine 300 CI MPR affinity purified antibody. Note the difference in labeling density. Magnification = 103,000 X, bar = 0.2 μm.

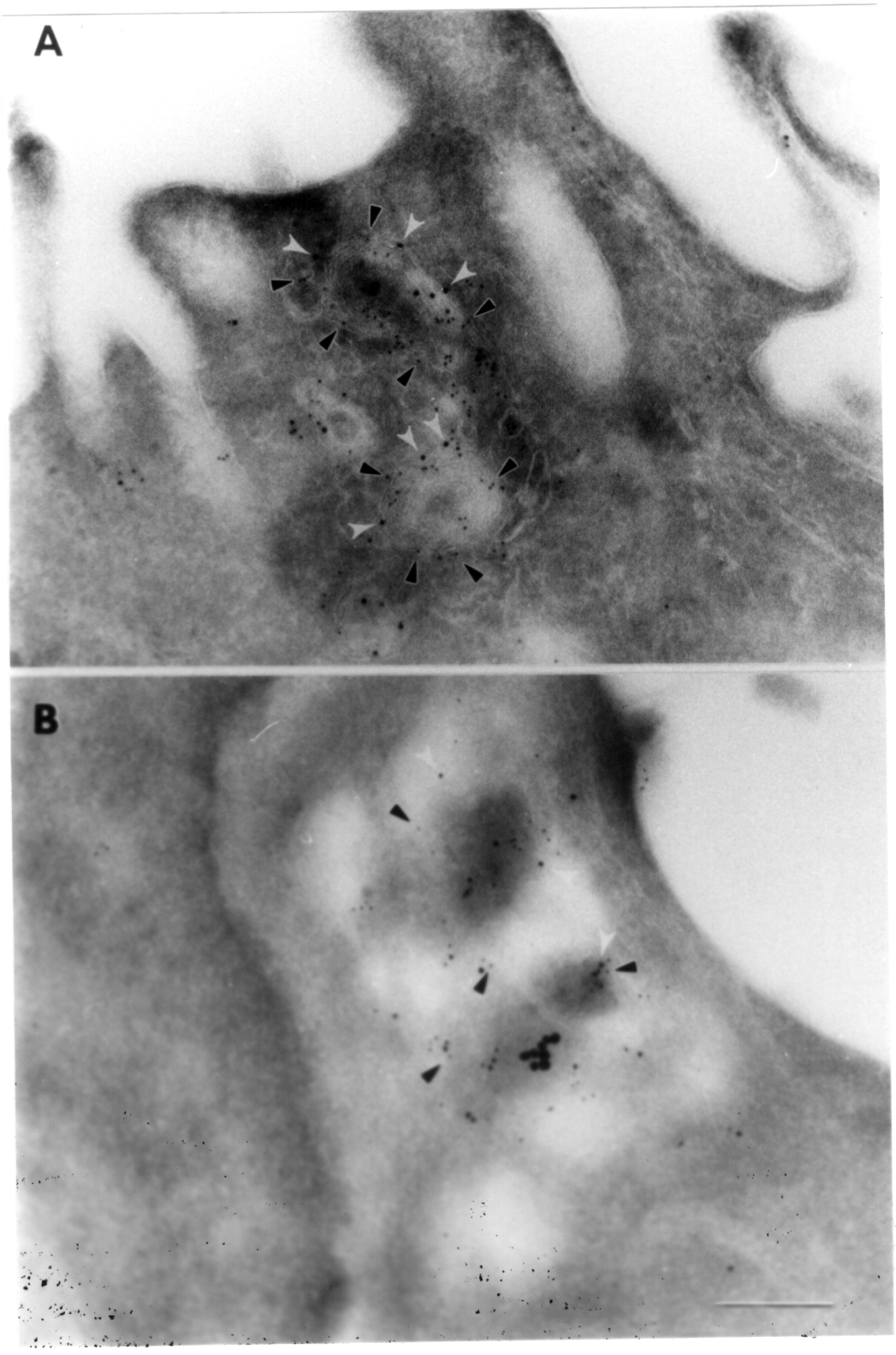
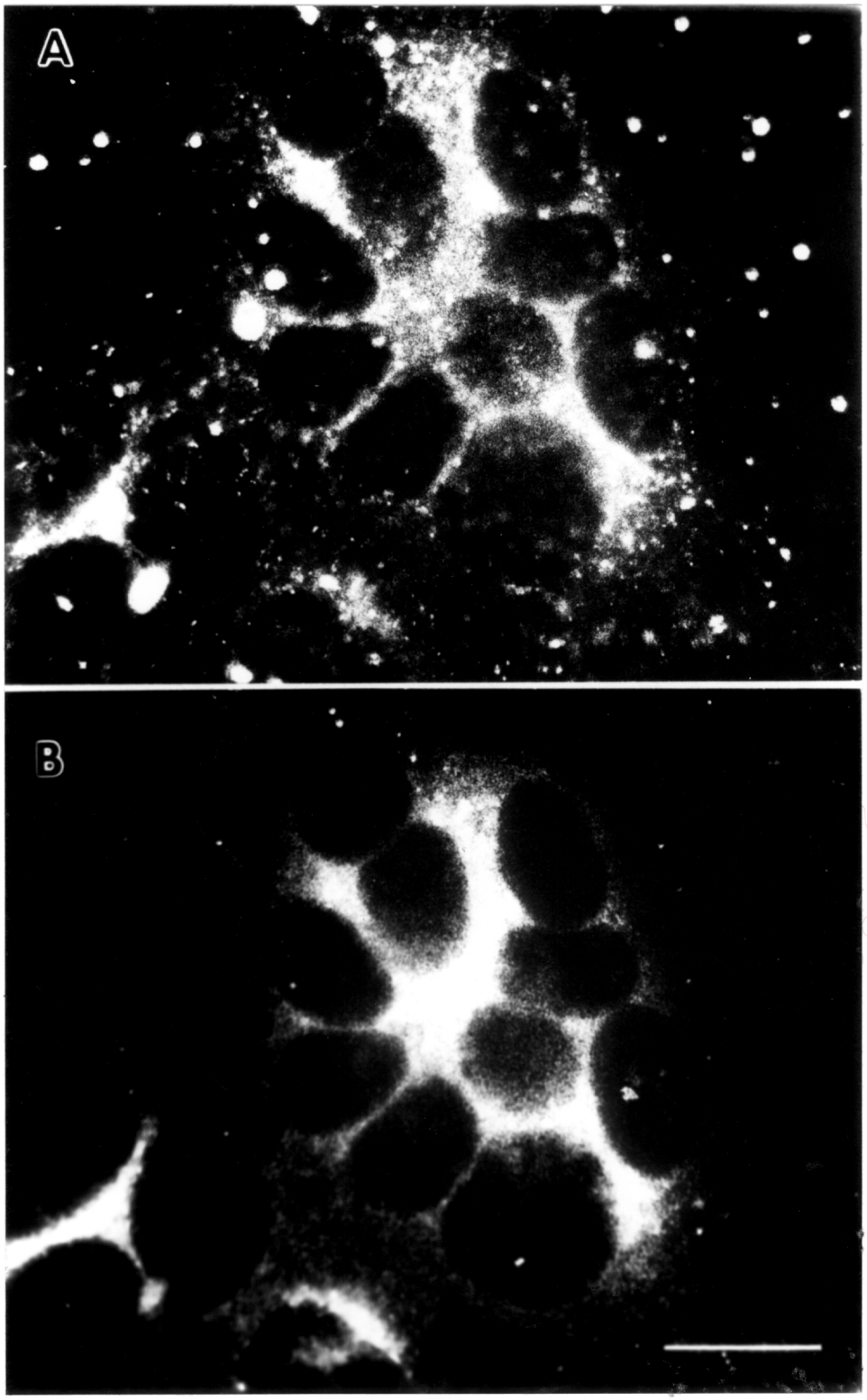

A
B

Fig. 4-8. Co-extensive distribution of PLC and dextran-positive endocytic compartment in NRK-3T3 syncytia. Three h post UV-inactivated virus mediated cell fusion, NRK-3T3 syncytia were incubated for 10 min with fixable rhodamine dextran and the dextran then chased for 50 min in marker-free media. Cells were then fixed and labeled with anti MPR and coumarin conjugated second antibody. Frame A: dextran (rhodamine channel). Frame B: MPR (coumarin channel). Magnification = 1300 X, bar = 20 μm.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Chapter 5. Comparative Kinetics of Resident and Transient Membrane Protein Transport Between Golgi Units in Fused Mammalian Cells Suggest Two Pathways For Transport of Golgi Proteins

5.1. Abstract

We have used the synchronized formation of a mixed cytoplasm upon heterokaryon formation cell fusion as a model for investigating the cisternal-specific transport of protein between neighboring Golgi apparatus. Rat NRK and hamster 15B cells were fused by UV-inactivated Sindbis virus and then incubated for various time periods in the presence of cycloheximide. The resident Golgi apparatus proteins, rat $GIMP_c$ and Golgp125, were localized with species-specific monoclonal antibodies. Immunofluorescent co-localization of rat and hamster Golgi membrane proteins was observed with a $t_{1/2}$ of 1.75 h; co-localization was accompanied by the acquisition of endoglycosidase H resistance by pre-existing Golgp125. Dispersal of the extended Golgi complex by nocodazole revealed that co-localization of resident Golgi proteins was due to intermixing of proteins in the same Golgi element rather than overlapping closely apposed of Golgi structures. The transient Golgi membrane protein, VSV-G protein, or its temperature sensitive derivative, tsO45-G protein, was transferred with a $t_{1/2}$ of ≤ 15 min from hamster to rat Golgi apparatus under permissive conditions. Co-localization of resident, but not transient Golgi membrane protein was concomitant with formation of a large extended Golgi complex; VSV-G protein exchange appeared to be between separate Golgi units in the heterokaryons. Little change in the number of cisternae/stack in cross sections of the Golgi apparatus was observed

upon cell fusion and in the extended Golgi complex the hamster resident protein remained localized to one side of the Golgi stack. Surprisingly, the morphological identity of the rat and hamster Golgi units appeared to be maintained in the hetero-karyons. Our results are consistent with two pathways for Golgi protein transport: a rapid, transient protein selective, vesicle-mediated pathway, and a slower, lateral diffusion pathway for mobile resident Golgi membrane proteins between interconnected Golgi units.

5.2. Introduction

The Golgi apparatus plays a central role in protein modification and protein sorting. It is an intermediate compartment between the endoplamic reticulum (ER) and the plasma membrane and consists of several subcompartments, namely the *cis*, *medial* and *trans* cisternae. Adjacent to the trans Golgi cisternae and perhaps continuous with it is the trans Golgi network, which is believed to be a major site of protein sorting (Griffiths, 1986). Golgi cisternae are functionally related to each other. Proteins are thought to transfer between *cis*, *medial* and *trans* Golgi cisternae by small vesicles which then fuse to the next cisterna. Both in *vivo* (Rothman, 1984 a,b) and in *vitro* (Balch et al., 1984; Braell et al., 1984; Orci et al., 1989), this appears to be a rapid, dissociative process ($t_{1/2}$ = ~10 min). It is also thought that stacks of Golgi cisternae observed to be separate in thin section may be connected to each other by a tubular network (Rambourg, 1990). This may promote protein exchange between Golgi stacks.

In interphase mammalian cells, the Golgi apparatus has been shown to be a dynamic structure. Reorientation of Golgi apparatus is observed during cell movement and during differentiation processes, such as the fusion of myoblasts to form myotubes (Tassin et al, 1985) or polarization of epithelial cells (Bacallao et al., 1989). In vitally stained cells, *trans* Golgi tubules rapidly extend and retract, forming and breaking interconnections with each other (Cooper et al., 1990). Upon cell fusion, the individual Golgi units remain, at least, transiently separate (Ho, et al., 1990; Pavlath et al., 1989; Rothman et, al. 1984 a; Tassin et al., 1985; Valtersson et al., 1990, and Xiao and Storrie, 1991). In homologous cell fusions, reorientation of the Golgi apparatus, accompanied by clustering and interconnections between Golgi units, occurs relatively rapidly between 30 min to 1 h post cell fusion (Ho et al., 1990; Xiao

and Storrie, 1991). These interconnections presumably form as a result of normal tubular dynamics between adjacent Golgi units.

The clustering and interconnection of Golgi apparatus in fused cells suggest a route for resident Golgi membrane protein intermixing between self-recognizing segments of the organelle, namely, lateral diffusion along interconnections. To test if resident protein intermixing between Golgi stacks occurs, we have assayed the distribution of resident rat and hamster Golgi membrane proteins in heterologous cell fusion experiments. The chief outcome of this work has been to show, by immunofluorescence and biochemical assays, that rat $GIMP_c$ (Yuan et al., 1987) and hamster Golgp125 (Cha et al., in preparation) intermix with a $t_{1/2}$ of ~1.75 h. This intermixing is concomitant with aggregation of the Golgi apparatus. The topological orientation of Golgp125 in the Golgi stack appeared to be maintained as well as the morphological properties of the organelle. By comparison, exchange of the transient Golgi membrane protein, VSV-G, was rapid ($t_{1/2}$ = ≤15 min) and selective; i.e., intermixing of resident proteins did not occur. These results raise the possibility that transport within the Golgi apparatus may occur by two different pathways.

5.3. Materials and Methods

Cells, Viruses and Antibodies

Cells were cultured in a 5% CO_2 incubator at 37°C. CHO cells were grown in F12 medium. CHO clones, 15B and Lec-1, were grown in αMEM medium. NRK cells were grown in DMEM medium. Culture media were supplemented with 10% fetal calf serum. For cell fusion, 3 X 10^6 15B cells were plated in 60 mm petri dishes 20 h prior to each experiment. In immunofluorescent staining experiments, 12 mm round coverslips were placed in the dishes prior to cell addition. VSV and Sindbis virus stocks were propagated in BHK cells. VSV mutant tsO45 was kindly given by Dr. Harvey Lodish, MIT, Cambridge, MA; ABL-70 is an rat monoclonal antibody, and a gift from Dr. Thomas August, Johns Hopkins, Baltimore, MD; $GIMP_c$ is a mouse monoclonal antibody, and a gift from Dr. Ignacio V. Sandoval, Madrid, Spain. Rabbit anti VSV G antibody was a gift from Dr. Thomas Kreis, EMBL, Germany. Second antibodies were purchased from Jackson ImmunoResearch Laboratories Inc, West Grove, PA. All of them have been adsorbed and are species-specific in their reaction with IgG.

VSV infection

Wild type or tsO45 VSV was incubated with 15B cells in αMEM plus 2% fetal calf serum for 1 h at room temperature with gentle rocking every 10 min. Free VSV was removed by rinsing the cells three times with warm medium. VSV concentration was sufficient to give bright immunofluorescent staining of Golgi apparatus at cell fusion. Infected cultures were maintained in αMEM supplemented with 5% fetal calf serum, 10 mM Hepes and 15 mM NH_4Cl (Rothman, 1984 a). The incubation temperatures

were 37°C for wild type and 39.5°C for tsO45. 15B cells infected with wild type or tsO45 VSV were incubated for 2.5 to 3 h at 37°C or 4.67 h at 39.5°C, respectively, before cell fusion. TsO45 infected cells were shifted to 31°C for 10 min to allow the G protein to transfer from ER to Golgi apparatus immediately before cell fusion.

Cell Fusion

3 X 10^6 NRK cells were added to the 15B culture. In some experiments, 15B cells were infected with VSV (wild type or tsO45 mutant) before the NRK cells were added (see above). The NRK and 15B cells were co-cultured for 2.5 to 3 hour. As fusogen, UV-inactivated Sindbis virus was bound to the 15B and NRK cells for 30 min on ice and cell fusion was initiated by shifting the pH to 5.0 (Ho et al., 1990).

Cycloheximide and Nocodazole Treatment

In all cell fusion experiments, cycloheximide (10 μ/ml) was present in the culture media post acid pH shift. In tsO45 experiments, tsO45 infected cultures were pre-treated with cycloheximide for 30 min before the cell fusion protocol. Nocodazole (10 μM) was added at different time points post-fusion, and cells were fixed 3 h later.

Immunofluorescent Staining

Cells were fixed, permeabilized with 0.1% saponin and immunofluorescently stained as described previously (Xiao and Storrie, 1991). The following antibody combinations were used: Golgp125 staining, ABL-70 first antibody and Texas Red conjugated goat anti-rat IgG second antibody; $GIMP_c$ staining, 15C8 first antibody and fluorescein conjugated goat-anti mouse IgG second antibody; VSV G staining, rabbit anti-VSV G first antibody and coumarin blue conjugated donkey anti-rabbit IgG second

antibody. For double labeling, permeabilized cells were first coincubated with two different first antibodies and then with two correspondent second antibodies. For triple labeling, permeabilized cells were first coincubated with three different first antibodies and then with three correspondent second antibodies. No cross reaction was observed between the second antibodies and the non-cognate first antibody.

Immunoperoxidase Electron Microscopy of Golgp125 in 15B cells and 15B-NRK Fused Cells

Cells were fixed for 1 h at 4°C and 1 h at room temperature in 2% formaldehyde, 0.075 M lysine, 0.01 M $NaIO_4$, in 0.0375 M phosphate buffer, pH 6.2 (MacLean and Nakane, 1974; Yuan et al., 1987). For 15B-NRk fused cells, 15B and NRK cells were fused and incubated at 37°C for 7 h post cell fusion protocol in the presence of cyclo-heximide before fixation. Cells were then permeabilized by incubating in 0.1% saponin in Buffer 1 (0.1% BSA in PBS) for 15 min at 37°C. Cells were incubated with first antibodies (rat ABL-70 antibody for Golgp125 and mouse 15C8 antibody for $GIMP_c$) for 1 h at room temperature, rinsed with Buffer 1, and incubated with HRP-conjugated second antibodies, either goat F(ab') fragment anti-rat IgG (for rat ABL-70 antibody) or goat anti-mouse IgG (for mouse 15C8 antibody) (Jackson ImmunoResearch, West Grove, PA). Cells were then fixed in 1.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4 containing 5% sucrose, and rinsed following the procedure of Brown and Farquar (1984). The peroxidase activity was localized by incubating the cells in 0.05% diaminobenzidine (DAB), 0.01% H_2O_2, in 50 mM Tris-HCL, pH 7.4, for 45 min at 4°C (Brown and Farquar, 1984). Cells were dehydrated in 70% ethanol, scraped off the culture dishes, and embedded in Spurr's Embedding Solution. Thin sections were prepared and examined in an electron microscope.

Quantitation of $GIMP_c$-Golgp125 and $GIMP_c$-VSV G Co-localization

At different time points following completion of the cell fusion protocol, cultures were fixed and immunofluorescently stained. Micrographs were taken sequentially in the fluorescein, Texas Red and coumarin channels using either a Zeiss 63 X planapo or 100 X Neofluar objective. Quantitation of doubly positive Golgi elements was by point counting as described (Griffiths et al., 1989; Deng, et al., 1991, in press)

[^{35}S-Methionine Incorporation and Immunoprecipitation of Golgp125

For [^{35}S]-methionine incorporation, cultures were washed three times with methionine free medium, and 0.5 mCi/ml [^{35}S]-methionine from a translation labeled mixture (EXPRE^{35}S^{35}S, NEN, DuPont, Boston, MA.) was added to the DMEM culture medium containing 44 μM non-radioactive methionine and supplemented with 10% dialyzed fetal calf serum. After 18 h incubation, [^{35}S]-methionine was removed by rinsing the cells with normal culture medium 3 times. For characterization of mature Golgp125 in unfused CHO and Lec-1 cells, cells were incubated in complete culture medium for 2 to 3 h as a chase. For characterization of Golgp125 in fused cells, 3 X 10^6 15B cells were labeled with [^{35}S]-methionine for 18 h as above. Cultures were rinsed with complete medium three times and 3 X 10^6 NRK cells were then added. The 15B and NRK cells were co-cultured for 3 h in 50% αMEM/50% DMEM supplemented with 10% fetal calf serum. Cell fusion was performed as above.

For immunoprecipitation, cultures were rinsed three times with detergent-free extraction buffer. Cell were removed by scrapping the culture dish gently with a rubber policeman, pelleted, lysed, and Golgp125 immunoprecipitated as described

previously by Hughes and August (1981) and as modified by Cha, Willingham and August (in preparation). The immunoprecipitants were dissolved in sample buffer and then applied to a 7.5% SDS-PAGE (Laemmeli, 1970). In some cases, dissolved immunoprecipitants were digested with endoglycosidase H before electrophoresis (Cha, Willingham and August, in preparation). Gels were soaked in Amplify (Amersham, Arlington Heights, IL.), dried and exposed to Kodak X-omat AR X-ray film (Kodak, Rochester, NY.) by fluorography (Bonner and Laskey, 1974). .

5.4. Results

Characterization of resident and transient Golgi membrane proteins in CHO and NRK cells

Golgp125 is a Golgi membrane protein identified in mouse 3T3 cells by a rat monoclonal antibody ABL-70 (Cha, Willingham and August, in preparation). It is located in the *cis* to *medial* cisternae of 3T3 Golgi apparatus, and is resistant to endoglycosidase H digestion. When wild type CHO cell extracts were reacted with ABL-70 antibody, an 125 kD polypeptide that exhibited resistance to endoglycosidase H digestion was identified (Fig. 5-1). In N-acetylglucosamine transferase I defective CHO clones (Lec-1 or 15B), the 125 kD polypeptide was fully sensitive to endoglycosidase H digestion (Fig. 5-1). To determine if the putative Golgp125 in CHO cells was a Golgi membrane protein, permeabilized cells were reacted with ABL-70 and Texas Red conjugated second antibodies. As shown in Fig. 5-2A, cells incubated with ABL-70 supernatant following fixation and permeabilization showed a perinuclear staining pattern characteristic of the Golgi apparatus. As expected for a membrane protein, a similar staining pattern was observed when the cells were permeabilized before fixation (data not shown). By immunoelectron microscopy, Golgp125 specific peroxidase reaction product was concentrated on one side of the Golgi stack (Fig. 5-2B). ABL-70 antibody exhibited no reactivity with NRK cells in immunofluorescence, immunoperoxidase or immunoprecipitation experiments (data not shown). We presume that the ABL-70 antigen identified in both mouse and hamster cells is the same polypeptide, Golgp125.

To introduce a transient membrane protein into the Golgi apparatus of CHO 15B cells, cells were infected with VSV or its tsO45 mutant. By double label immunofluo-

rescent staining, VSV-G accumulation in the Golgi apparatus was most pronounced 2.5 to 3 h post infection (Fig. 5-2C); little, if any, staining was detected in ER or at the plasma membrane. This infection period was used in all experiments with wild type VSV. With the virus dilution used, the amount of VSV-G protein per cell varied considerably (compare Fig. 5-2A,C). For maximal tsO45-G protein accumulation in the Golgi apparatus, 15B cells were cultured for 4.5 h post infection at 39.5°C , the non-permissive condition for the mutant G protein transport from ER to Golgi, followed a 10 min chase at 31°C. Consistent with previous work (Kreis, 1986), after the 10 min temperature shift, tsO45-G protein specific immunofluorescence was present in the Golgi apparatus with little detectable staining in ER (data not shown, see also Fig. 5-13C). These conditions were used for all subsequent tsO45 infections.

$GIMP_c$ is an 130 kD Golgi membrane protein identified in rat NRK cells by mouse monoclonal antibody 15C8 (Yuan et al., 1987). It is located in the *cis* to *medial* cisternae of the NRK Golgi. By immunoelectron microscopy, $GIMP_c$ specific peroxidase reaction product was found predominantly in perinuclear, tubulovesicular structures and in some cases in stacked cisternae (Fig. 5-3). The tubulovesicular Golgi apparatus of NRK cells appeared morphologically distinct from the stacked Golgi apparatus of 15B cells (for quantitation, see Table I). The 15C8 antibody showed no reactivity with CHO cells (data not shown).

Kinetics of resident Golgi membrane protein intermixing in NRK-15B syncytia

To ask if resident Golgi membrane protein intermixing between Golgi stacks occurs, we fused $GIMP_c$-positive NRK cells and Golgp125-positive 15B cells, and assayed for the distribution of the proteins by double label immunofluorescence. As

shown in Fig. 5-4A and B, 20 min after the initiation of cell fusion, $GIMP_c$ and Golgp125 were localized in non-overlapping, relatively compact perinuclear structures. After 1 to 7 h post-fusion, Golgi apparatus in the heterokaryons were congregated to form an extended Golgi complex in association with the clustered nuclei (Fig. 5-4C-H). This extended complex contained contributions from both the rat and hamster parent cells. Initially, most Golgi elements in the extended complex were positive for either $GIMP_c$ or Golgp125, with only limited co-localization of the proteins being observed (arrowheads, Fig. 5-4C,D). With time progressively greater, co-localization of $GIMP_c$ and Golgp125 was found (Fig. 5-4E-H). Quantitatively, co-localization of $GIMP_c$ and Golgp125 had a half time of 1.75 h and after 7 h, more than 90% of Golgi elements were positive for both proteins (Fig. 5-5).

To investigate if co-localization of $GIMP_c$ and Golgp125 in the heterokaryons was due to an actual intermixing of molecules or an inability of the immunofluorescence technique to resolve closely apposed Golgi elements, the Golgi was dispersed by adding nocodazole at different times post cell fusion. In the absence of nocodazole, 5 h post cell fusion, perinuclear, extended Golgi complexes were observed and the Golgi elements within the complex were frequently positive for both $GIMP_c$ and Golgp125 (Fig. 5-6A,B). Nocodazole addition resulted in dispersal of the extended Golgi complex throughout the syncytia (Fig. 5-6C,D). When nocodazole was added 5 h post fusion, $GIMP_c$ and Golgp125 were frequently co-localized in the dispersed Golgi elements; some elements stained equally brightly for both protein (black arrowheads), others stained more brightly for $GIMP_c$ or Golgp125 (white arrowheads). Quantitively, a progressive increase in doubly-positive Golgi elements following nocodazole treatment was observed as the time after cell fusion increased (Fig. 5-7), consistent with the previously cited kinetics of $GIMP_c$-Golgp125 co-localization in

the syncytia. At early times post fusion, some increase in co-localization occurred during the 3 h nocodazole treatment. Presumably this was due to the time required for nocodazole to disrupt microtubules. We conclude that $GIMP_c$ and Golgp125 co-localized in the cell syncytia over time.

As a biochemical assay for the intermixing of NRK and 15B Golgi apparatus, the sensitivity of Golgp125 to endoglycosidase H digestion in the heterokaryons was determined. As shown in Fig. 5-8, Golgp125 in newly fused cells was endoglycosidase H sensitive as was Golgp125 in 1 h old syncytia. In 4 h old syncytia, the protein appeared resistant to endoglycosidase H digestion, consistent with NRK correction of the 15B N-acetylglucosamine transferase I defect.

Morphology of Golgi apparatus in fused cells

NRK and 15B Golgi apparatus can be distinguished from each other on the basis of their morphology (tubulovesicular versus stacked, Table 1). To determine whether or not two major morphological classes of Golgi apparatus were present in the syncytia, cultures containing approximately equal numbers of NRK and 15B cells were fused, fixed and processed for immunoelectron microscopy 7 h post cell fusion. >95% of the cells were fused, and the syncytia were stained for Golgp125. Two morphological classes of Golgp125-positive Golgi structures were observed in the syncytia (Fig. 5-9, Table 1). In the most common class, ~70% of the total, the Golgi appeared as a set of stacked cisternae with the Golgp125-specific staining on one side of the stack. In the second class, ~30% of the total, the Golgi appeared as a tubulovesicular structure which was positive for Golgp125. Presumably the Golgi displaying a stacked arrangement of cisternae are of 15B origin, and those displaying a tubulovesicular arrangement are of NRK origin.

Kinetics of transient Golgi membrane protein intermixing in NRK-15B syncytia: native VSV G protein

As described earlier, for VSV infected 15B cells, the bulk of VSV-G protein 2.5 h after infection appeared to be present in the Golgi apparatus. Therefore, it may be considered as a transient Golgi membrane protein. To compare the kinetics of exchange of VSV-G protein between Golgi apparatus with that of Golgp125, 15B cells were infected and then fused with NRK cells. Cycloheximide was added at the end of the acid pH shift used to initiate cell fusion, and cells were then incubated for 10 min to allow cell fusion to proceed. Under our experimental conditions at 37°C, 10 to 15 min was required for cell fusion. At the end of the 10 min cell fusion period, VSV-G protein was found only in 15B Golgi apparatus, Golgp125-positive structures. No overlapping of the distribution of rat ($GIMP_c$) and hamster (Golgp125) Golgi apparatus was observed (Fig. 5-10A, B, C). Cells were then incubated at either 20°C or 37°C. At 20°C, congregation of Golgi apparatus and nuclei is inhibited (Ho et al., 1990). After 20 min further incubation at 20°C, VSV-G protein co-localized with both Golgp125 and $GIMP_c$ (Fig. 5-10D,E,F), indicating the transfer of VSV-G protein into NRK Golgi. At the end of 45 min incubation at 20°C, this co-localization was more extensive (Fig. 5-10G,H,I). As shown quantitatively in Fig. 5-11, VSV-G protein co-localization with $GIMP_c$ was much more rapid than that of Golgp125 with $GIMP_c$. At 37°C, VSV-G protein co-localization with $GIMP_c$ was even more rapid. When following the 10 min cell fusion period, syncytia were incubated at 37°C for an additional 10 min, the majority of $GIMP_c$-positive structures were also positive for VSV-G protein, and little co-localization of $GIMP_c$ and Golgp125 was observed (Fig. 5-12). Rapid co-localization of VSV-G protein with $GIMP_c$ was seen even with

highly diluted virus infection of 15B cells, indicating that the rapid detection of VSV-G protein/GIMP$_c$ but not Golgp125/GIMP$_c$ co-localization was not due to the high concentration of VSV-G protein relative to Golgp125.

Kinetics of transient Golgi membrane protein intermixing in NRK-15B syncytia: tsO45-G protein

To synchronize VSV-G protein localization in the Golgi, 15B cells were infected with the tsO45 mutant of VSV for 4.5 h at 39.5°C and then shifted for 10 min to 31°C, permissive conditions for tsO45 G protein transport from ER to Golgi. In these experiments, cycloheximide was added at 4.0 h post cell infection. At the end of the temperature shift, co-cultured NRK and tsO45 infected-15B cells were incubated briefly at an acid pH to initiate their fusion. Cells were then cultured at 39.5°C, fixed at various time points and stained for GIMP$_c$, Golgp125, and tsO45-G protein. At zero time, tsO45-G protein was found in Golgp125-positive 15B Golgi, but not in GIMP$_c$-positive NRK Golgi (Fig. 5-13A,B,C). Thirteen min post acid pH shift, a significant fraction of GIMP$_c$-positive Golgi were also positive for tsO45-G protein (Fig. 5-13D,E,F). Little co-localization of GIMP$_c$ and Golgp125 was seen at this time point. By 23 min post acid pH shift, more than half of the GIMP$_c$-positive structures were tsO45-G protein positive (Fig. 5-13G,H,I, Fig. 5-14). During the 39.5°C post fusion incubation, some G protein had appeared at the syncytia plasma membrane as suggested by the background G protein staining at the late time points. At all time points, the extent of tsO45-G protein co-localization with GIMP$_c$ was much greater than that of GIMP$_c$ with Golgp125 (Fig. 5-14). Rapid co-localization of tsO45-G protein with GIMP$_c$ was seen even with high dilution of infecting virus (data not shown).

5.5. Discussion

We have used the synchronized rearrangement of Golgi apparatus upon heterologous cell-cell fusion as a model for investigating the cisternal-specific exchange of proteins between neighboring Golgi units. The aim of this study was to find general answers to two questions related to the transport properties of Golgi membrane proteins. Firstly, are resident Golgi membrane proteins mobile and hence able to exchange between neighboring Golgi units, albeit, perhaps slowly? Secondly, in a kinetic sense, what are the differences between resident and transient Golgi membrane protein transport? We find that resident Golgi membrane proteins do intermix between Golgi units and hence must be mobile. Moreover, we find that transport of resident proteins between Golgi units is slow in comparison to the transient protein, VSV-G protein, and concomitant with organelle congregation; it is likely by a different mechanism.

In all experiments, Golgi protein intermixing was assessed in cycloheximide treated heterokaryons formed by fusion of rat and hamster cells. The NRK-15B cell pairing was dictated by the availability of species-specific antibodies to resident Golgi membrane proteins. The 15C8 mouse monoclonal antibody (Yuan et al., 1987) reacted with the NRK cell line but not with the CHO cell line or its N-acetylglucosamine transferase I mutants (15B and Lec-1). Similarly, ABL-70 rat monoclonal antibody (Cha, Willingham and August, in preparation) reacted with CHO but not NRK. The antibodies identified unique proteins, $GIMP_c$ (Yuan et al., 1987) and Golgp125 (current work, Cha, Willingham and August, in preparation) respectively. VSV-G protein was localized by a rabbit polyclonal antibody preparation. The Golgi localization of VSV-G protein and its tsO45 mutant was controlled by the infection protocol and chase period prior to the formation of fused cell syncytia. In most experiments,

$GIMP_c$, Golgp125, and VSV-G protein were localized with respect to each other by double or triple label immunofluorescence. In some experiments, the proteins were localized by immunoperoxidase electron microscopy. The more quantitative ultrastructural approach of double label immunogold localization of $GIMP_c$ and Golgp125 was not possible. In preliminary experiments, Golgp125 proved to be insufficiently abundant to permit its localization by immunogold staining of cryosections (Deng, Griffiths and Storrie, unpublished observations). The localization of Golgp125 with respect to N-acetylglucosamine transferase I activity, present in wild type NRK Golgi, was also inferred from the electrophoretic behavior of endoglycosidase H treated immunoprecipitants of Golgp125.

As scored by these assays, the intermixing within Golgi elements of the resident membrane proteins, $GIMP_c$ and Golgp125, was at all temperatures slow, $t_{1/2} = \geq 1.75$ h, while intermixing of VSV-G protein or its tsO45 mutant between Golgi units was rapid, $t_{1/2} = \leq 15$ min to 30 min (Table 2). This second result is consistent with earlier *in vivo* and *in vitro* observations by Rothman and colleagues (Rothman, 1984,a,b; Braell et al., 1984). Intermixing of resident proteins was accompanied by the reorientation and congregation of Golgi units to form an extended Golgi complex about the clustered nuclei. Similar congregation of a second perinuclear organelle, the pre-lysosomal compartment (PLC), has been reported previously (Deng, Griffiths and Storrie, 1991, in press). $GIMP_c$ and Golgp125 appeared to intermix within the same membrane segments; $GIMP_c$ and Golgp125 co-segregated when Golgi apparatus was dispersed by nocodazole treatment of the syncytia. Intermixing or transport of VSV-G protein from hamster to rat Golgi apparatus was between apparently separate Golgi units at all temperatures including 20°C, a temperature at which clustering of Golgi to form an extended Golgi complex is inhibited (current work, Ho et

al., 1990). Surprisingly, in thin sectioned syncytia, even 7 h after cell fusion, two major morphologically recognizable, Golgp125-positive classes of Golgi units could be distinguished, a tubulovesicular class and a stacked cisternal class corresponding to NRK and 15B Golgi respectively. This last observation suggests that the local, intercisternal morphological characteristics of the interphase Golgi apparatus are controlled by very stable associations. Morphologically, Golgp125 maintained its asymmetric distribution within the stacked cisternae of NRK-15B heterokaryons, consisted with intermixing being cisternal-specific.

Based upon these results and other work (Cooper et al., 1990, Ho et al., 1990 Pavlath et al., 1989; Xiao and Storrie, 1991), we suggest that intermixing of resident Golgi membrane protein is the outcome of lateral diffusion along tubular connections, i. e., direct physical continuities between closely apposed Golgi structures. In cell syncytia, reorientation and congregation of individual Golgi apparatus may place Golgi units in close proximity (current work, Ho et al., 1990; Pavlath et al; Tessin et al., 1985). Only under these conditions, i. e., close apposition, do we observe molecular intermixing of donor and recipient resident Golgi proteins. We suggest that close proximity leads to the formation of direct interconnections between previously separated Golgi units. Cooper et al. (1990) have shown *in vivo* that *trans*-Golgi tubules rapidly make and break interconnections with each other. In homologous cell fusions, we have shown that interconnections may form between proximal Golgi units within 30 min to 1 h of cell fusion (Xiao and Storrie, 1991). In the event that the individual Golgi apparatus stay separate, they may function independently within the syncytium (Pavlath et al., 1989; Valtersson et al., 1990).

We propose that there are two separate routes for the transport of Golgi proteins, with one being cisternal-specific lateral diffusion and the other being dissociative

vesicle-mediated transport. We suggest that transient proteins are transported within the Golgi apparatus by a dissociation process, which is presumably vesicle-mediated. This suggestion is in full agreement with the previous conclusions of Rothman and colleagues (Rothman 1984 a, b). Based on the present results, both resident and transient Golgi membrane proteins must be mobile. Further direct experiments will be necessary to measure the actual lateral mobility of these membrane proteins.

5.6. References

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Table 1. Morphological characteristics of Golgi apparatus in NRK cells, 15B cells, and NRK-15B syncytia

	Number of Golgi counted	% Vesicular	% Stacked	Average number of cisternae in stacks (± 1 standard error)
15 B Cells	28	7	93	4.2 ± 0.3
NRK Cells	33	76	24	2.5 ± 0.3
Fused Cells	40	35	65	3.4 ± 0.2

Table 2. Estimated half time for intermixing of resident and transient Golgi membrane proteins in fused NRK-15B cells

	Temperature		
	20°C	37°C	39.5°C
Resident proteins Golgp125/GIMP$_c$	~3.75 h	~1.75 h	~0.86 h
Transient protein VSV/ts045-G protein	~0.50 h	≤0.25 h	~0.25 h

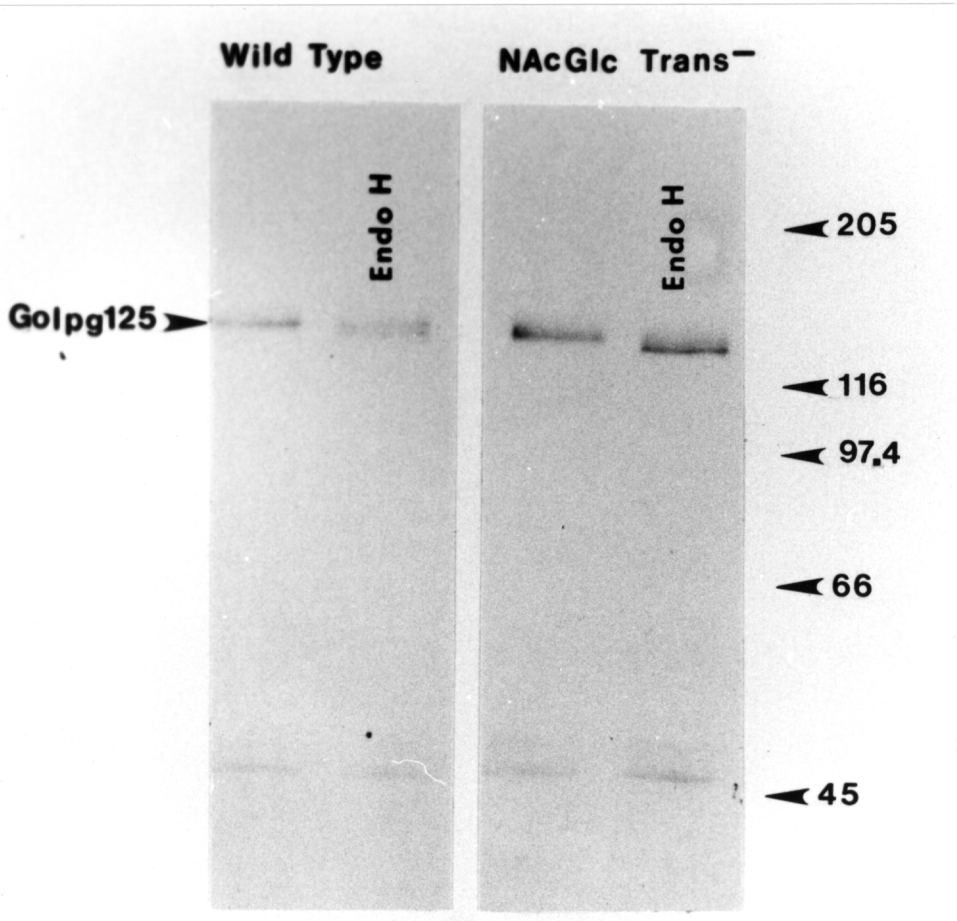
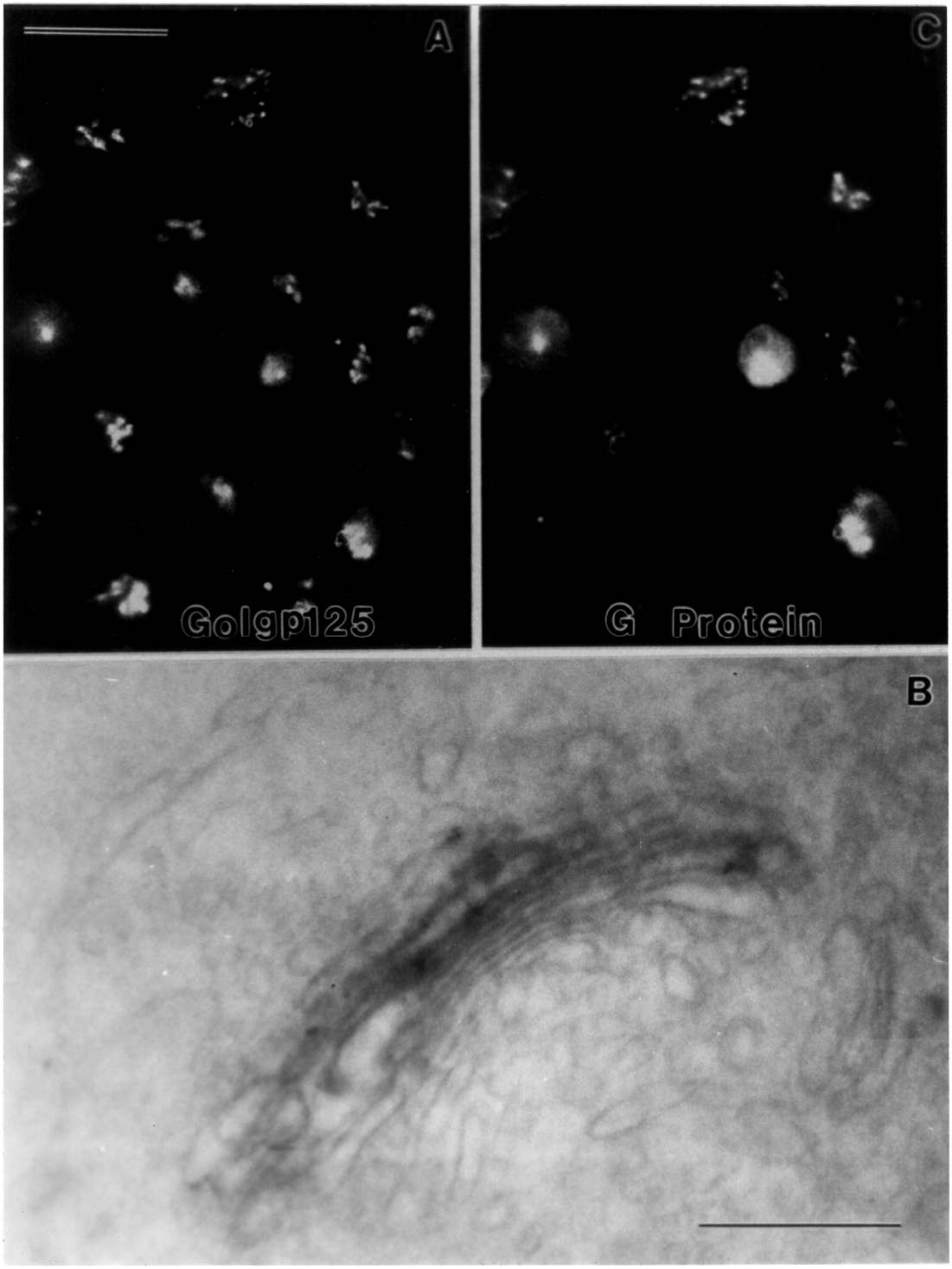


Fig. 5-1. Characterization of Golgp125 from CHO and Lec-1 cells. The total protein was labeled by overnight incubation of cultures with [^{35}S]-methionine. Cells were lysed, and Golgp125 was immunoprecipitated with the rat ABL-70 monoclonal antibody. The precipitants were dissolved in sample buffer. Half of the samples were digested with endoglycosidase H for 18 h at 37° C. Samples were applied to a 7.5% SDS-PAGE. The calculated molecular weight of Golgp125 is 125 kD.

Fig. 5-2. Localization of Golgp125 (A,B) and VSV-G protein (C) in 15B cells. In frames A and C, 15B cells were infected with wild type VSV and cells were fixed 2.5 h post infection. Cells were double stained for Golgp125 (rat ABL-70 antibody and Texas Red conjugated second antibody) and VSV-G protein (rabbit anti-VSV-G protein antibody and coumarin blue conjugated second antibody). In frame C, 15B cells were fixed permeabilized and stained with ABL-70 and peroxidase conjugated second antibody. Peroxidase activity was localized by the DAB reaction. Cells were then processed for electron microscopy. Frame A and C: magnification = 941 X, Bar = 20 μm; Frame B, magnification = 88,000 X, Bar = 0.3 μm.


A
Golgp125
C
G Protein
B

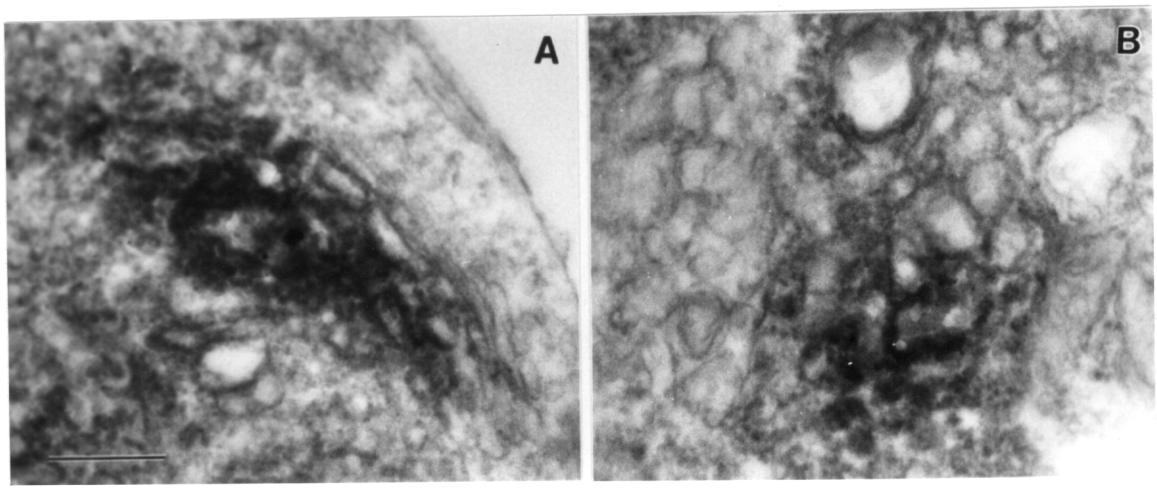
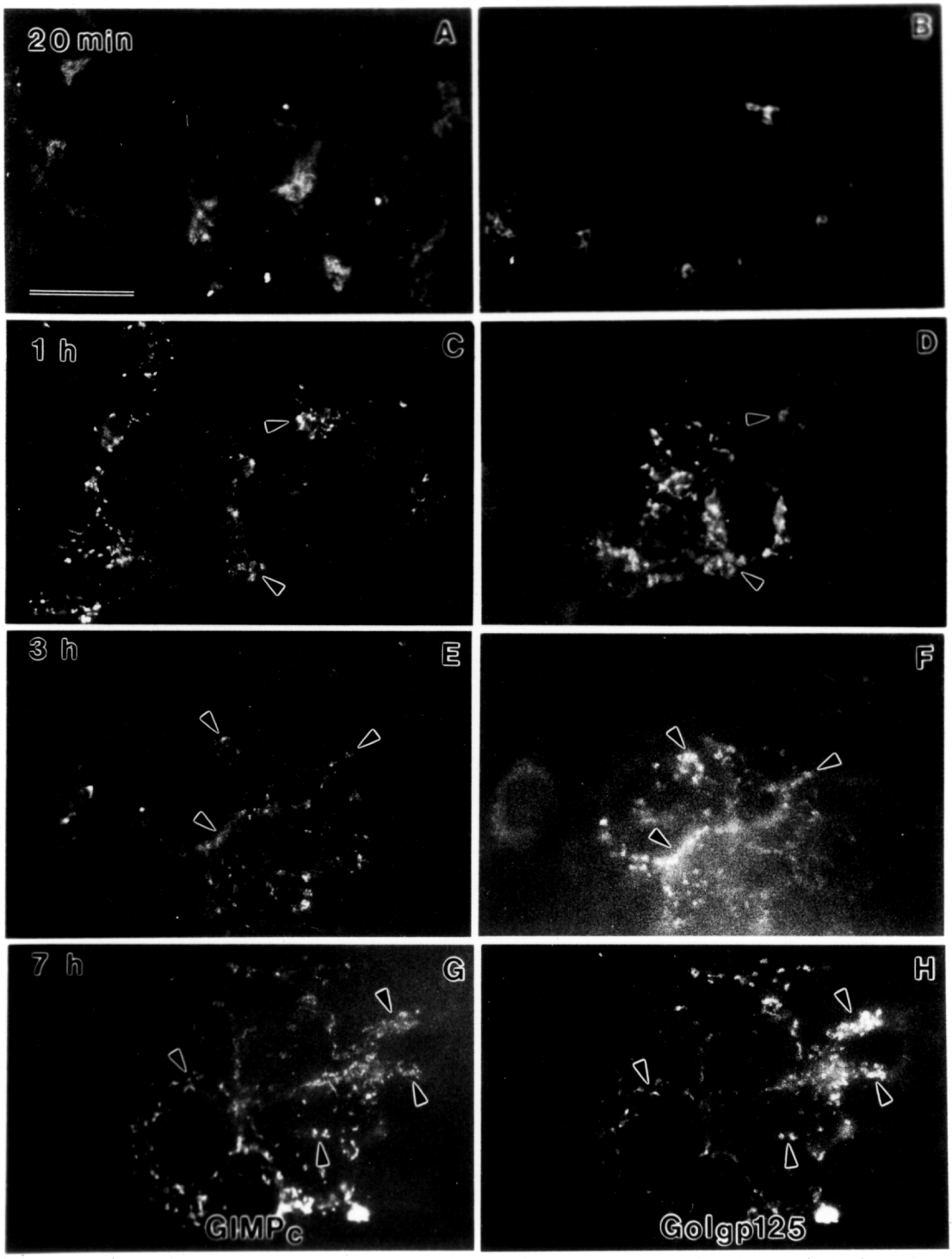


Fig. 5-3. Immunoperoxidase localization of $GIMP_c$ in NRK cells. NRK cells were fixed, permeabilized and stained with mouse 15C8 antibody and peroxidase conjugated second antibody. Peroxidase activity was localized by DAB reaction. Cells were processed for electron microscopy. Frame A shows the example of the $GIMP_c$-positive tubulovesicular Golgi. Frame B shows the example of the $GIMP_c$ stacked Golgi. Magnification = 54,000, Bar = 0.3 μm.

Fig. 5-4. Immunofluorescent localization of $GIMP_c$ and Golgp125 in NRK-15B fused syncytia. NRK and 15B cells were fused in the presence of cycloheximide and fixed at various times. Cells were permeabilized and double stained for $GIMP_c$ (15C8 antibody and fluorescein conjugated second antibody, left column, A,C,E,G) and for Golgp125 (ABL-70 antibody and Texas Red conjugated second antibody, right column, B,D,F,H). For each time set (A and B, C and D, E and F, G and H), micrographs were taken in the same focal plane but in different fluorescent channels. Arrowheads point to the Golgi elements that are positive for both $GIMP_c$ and Golgp125. Magnification = 927 X, Bar = 20 μm.


20 min
A
B
1 h
C
D
3 h
E
F
7 h
G
H
GIMPc
Golgp125

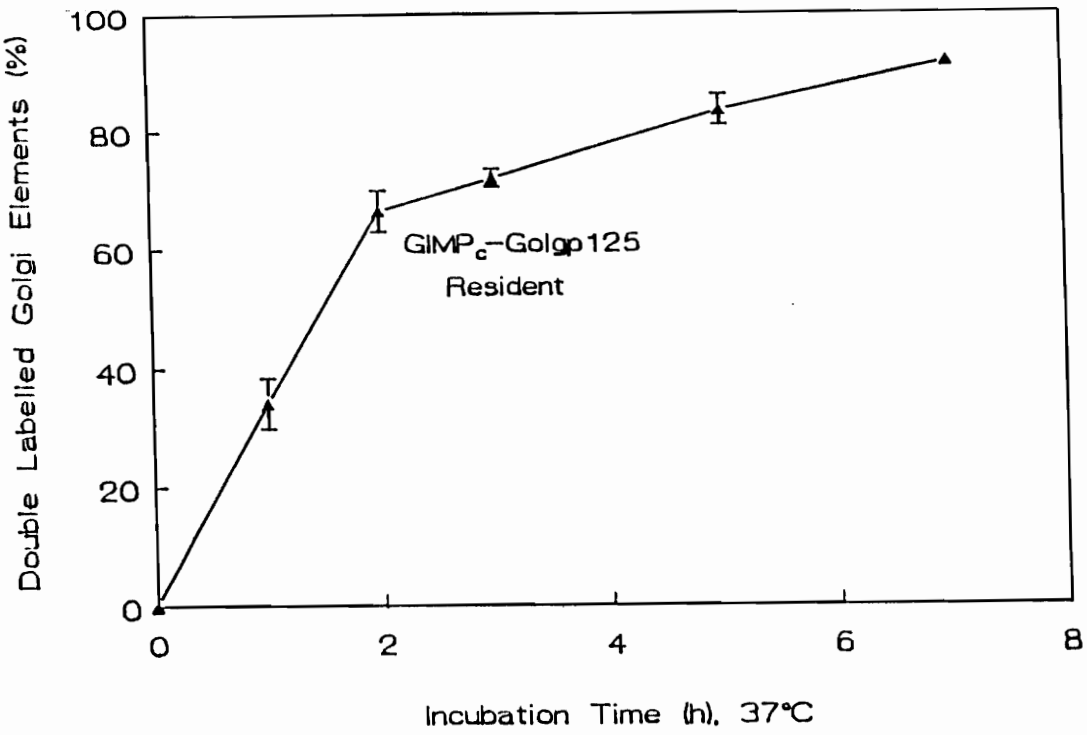


Fig. 5-5. Kinetics of Golgi resident membrane protein intermixing. NRK and 15B cells were fused in the presence of cycloheximide and fixed at different times. Cells were double labeled for GIMP$_c$ and Golgp125 as in Fig. 5-4. Quantitation of double labeling was as described in materials and methods. About 15 sets of micrographs were scored for each time point.

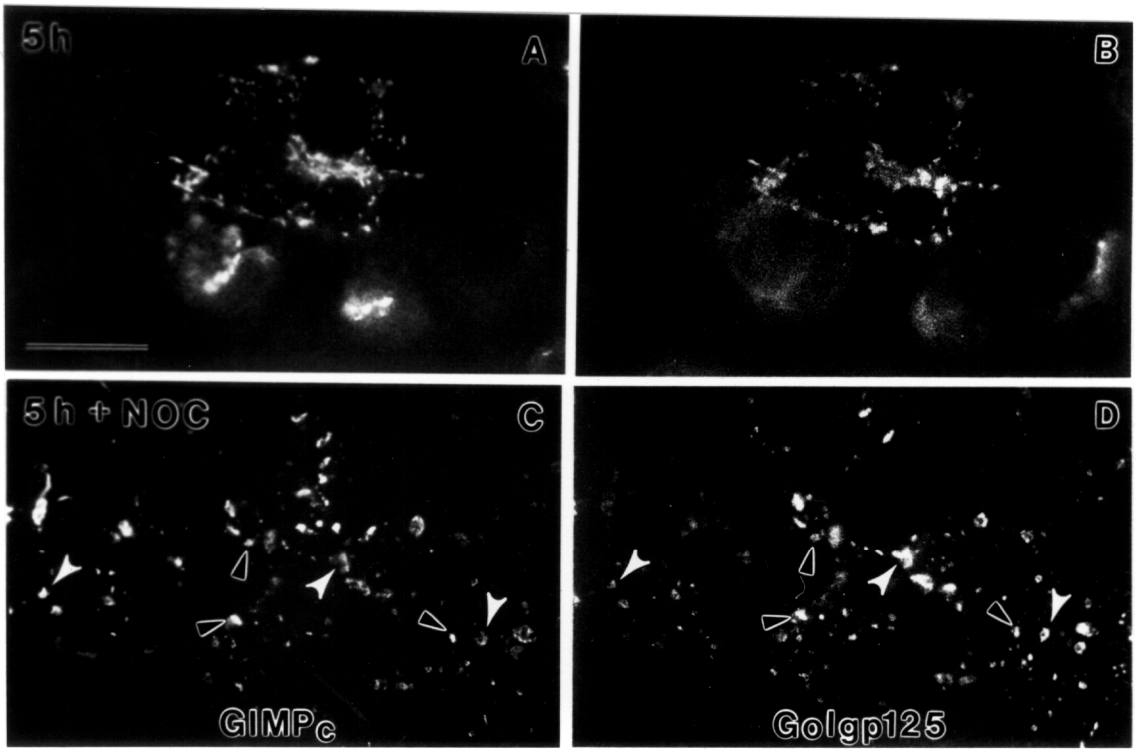


Fig. 5-6. Effect of nocodazole treatment on the immunofluorescent double labeling of $GIMP_c$ and Golgp125 in NRK-15B syncytia. NRK and 15B Cells were fused in the presence of cycloheximide and incubated for 5 h. In A, B, cells were fixed immediately. In C,D, 10 μM of nocodazole was added to the cultures 5 h post fusion and cells were incubated for another 3 h in the presence of nocodazole and cycloheximide. Cells were double stained for $GIMP_c$ (A,C) and Golgp125 (B,D) as described in Fig. 5-4. Micrographs A and B, C and D were taken in the same focal plane but in different fluorescent channels. Arrowheads point to the Golgi elements positive for both $GIMP_c$ and Golgp125. Some of these double positive Golgi elements stained more brightly for one protein then the other (white arrowheads) and some stained equally brightly for both (black arrowheads). Magnification = 913 , Bar = 20 μm.

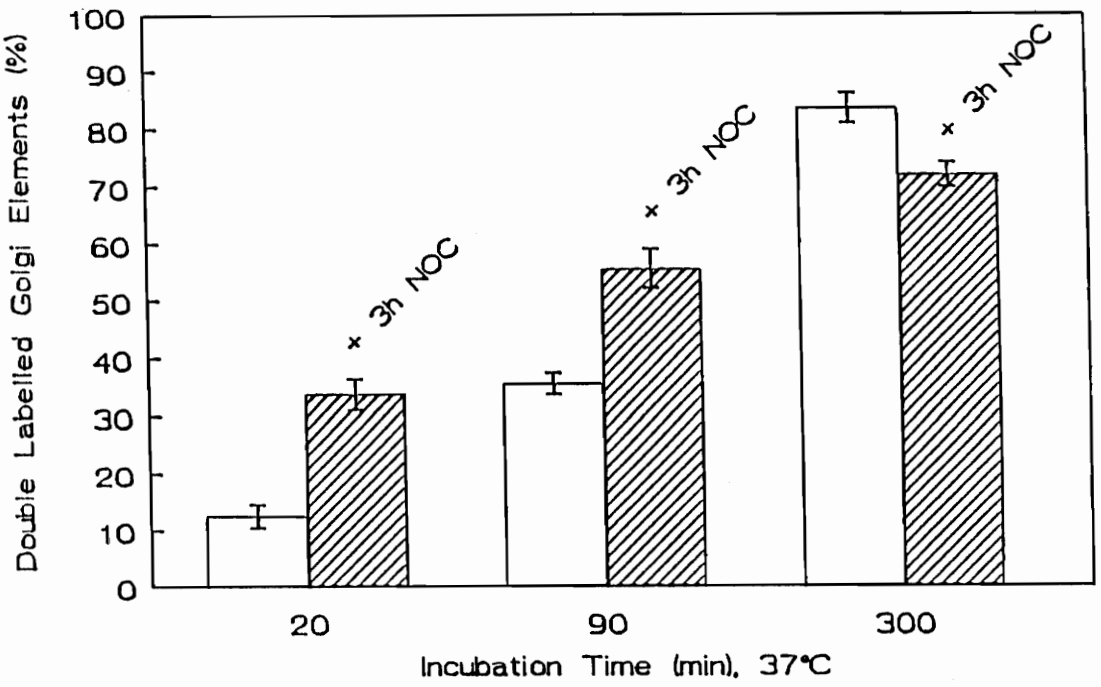


Fig. 5-7. Effect of nocodazole on the kinetics of resident Golgi membrane protein co-localization. In the control (open bars), NRK and 15B cells were fused in the presence of cycloheximide and fixed at different times. In the nocodazole treatment (hatched bars), NRK and 15B cells were fused in the presence of cycloheximide and nocodazole was added at different time points. Nocodazole treated syncytia were incubated for an additional 3 h before fixation. Fixed cells were double stained for $GIMP_c$ and Golgp125 as described in Fig. 5-4. Double labeled Golgi elements was scored as described in material and methods. For each time point, about 15 sets of micrographs were scored.

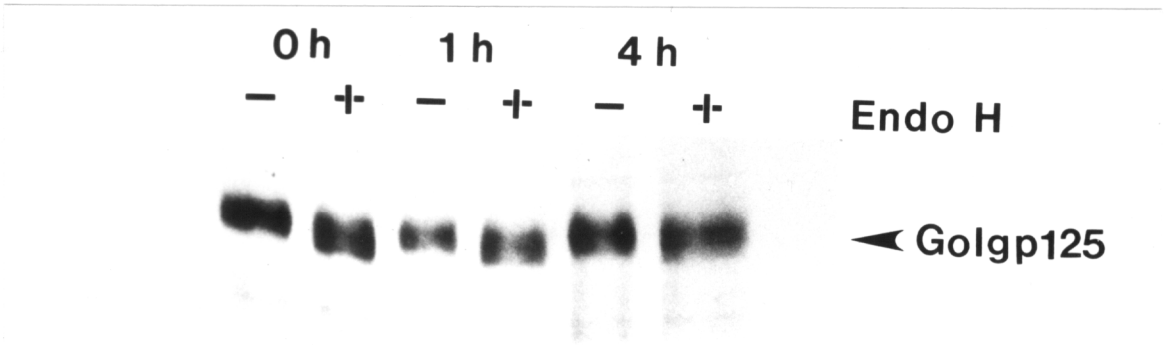


Fig. 5-8. Effect of cell fusion of the endoglycosidase H suseptibility of Golgp125. 15B cells labeled with [^{35}S]-methionine for 18 h were fused with NRK cells in the presence of cycloheximide. At different times after the fusion, cells were lysed and Golgp125 was immunoprecipitated. The precipitants were dissolved in sample buffer and processed for endoglycosidase H digestion as described in Fig. 5-1. The samples were applied to a 7.5% SDS-PAGE.

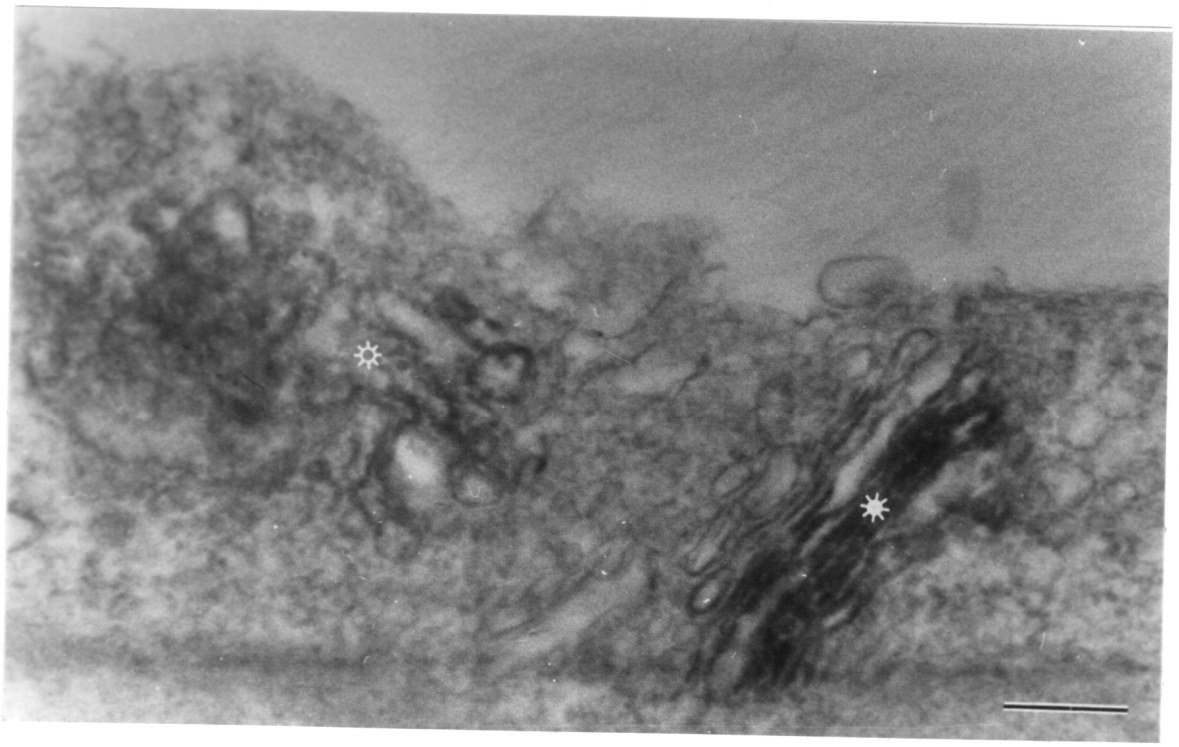
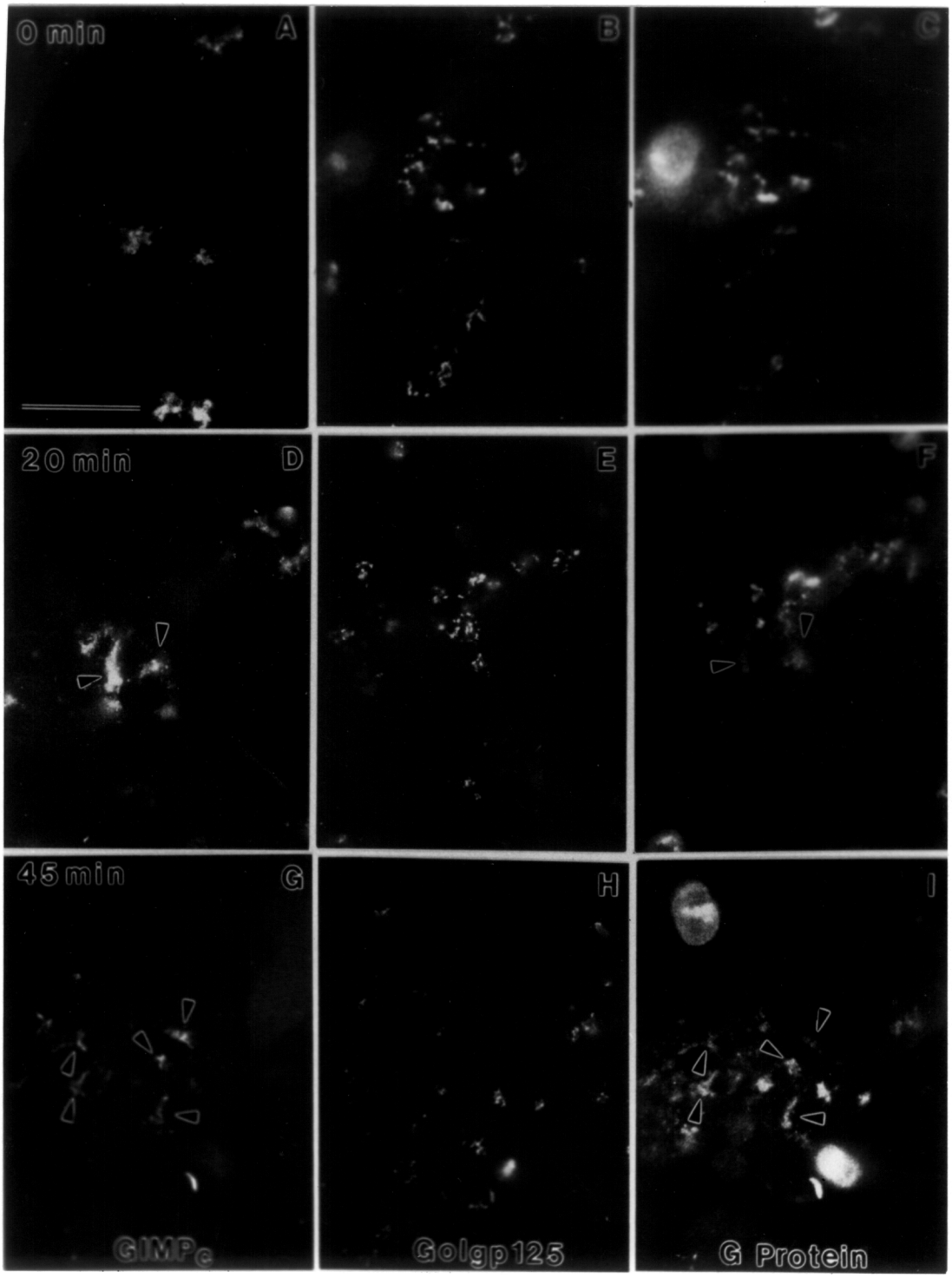


Fig. 5-9. Morphology of the Golgi apparatus in fused NRK-15B cells. NRK and 15B cells were fused in the presence of cycloheximide and fixed 7 h post cell fusion. The cells were stained for Golgp125 with ABL-70 and peroxidase conjugated second antibody. Cells were then processed for DAB immuno electron microscopy. The electron dense deposits indicate the Golgp125 localization. The Golgi apparatus appeared to be either tubulovesicle (hollow star) or stacked (filled star). Golgp125 was located on one side of the Golgi stack. Magnification = 43,000, Bar = 0.3 μm.

Fig. 5-10. Triple immunofluorescent labeling of $GIMP_c$, Golgp125 and VSV-G protein in NRK-15B fused cells. NRK and 15B cells were fused in the presence of cycloheximide 2.5 h after infection of 15B cells wild type VSV. Immediately after the fusion protocol, cells were incubated at 37° C for 10 min to permit cell fusion, and then the cells were shifted to 20° C. Cells were fixed at different time points after the 20° C temperature shift and stained for $GIMP_c$, Golgp125, and VSV-G protein. $GIMP_c$ was localized with mouse 15C8 antibody and fluorescein conjugated second antibody (left column, A,D,G); Golgp125 with rat ABL-70 antibody and Texas Red conjugated second antibody (middle column, B,E,H); VSV-G protein with rabbit anti VSV-G antibody and coumarin blue conjugated second antibody (right column, C,F,I). For each time set (A,B,C; D,E,F; G,H,I), micrographs were taken in the same focal plane but in different fluorescent channels. Arrowheads point to the Golgi apparatus which are positive for both $GIMP_c$ and VSV-G protein. Magnification = 1,051 X, Bar = 20 μm.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GIMPc
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G Protein

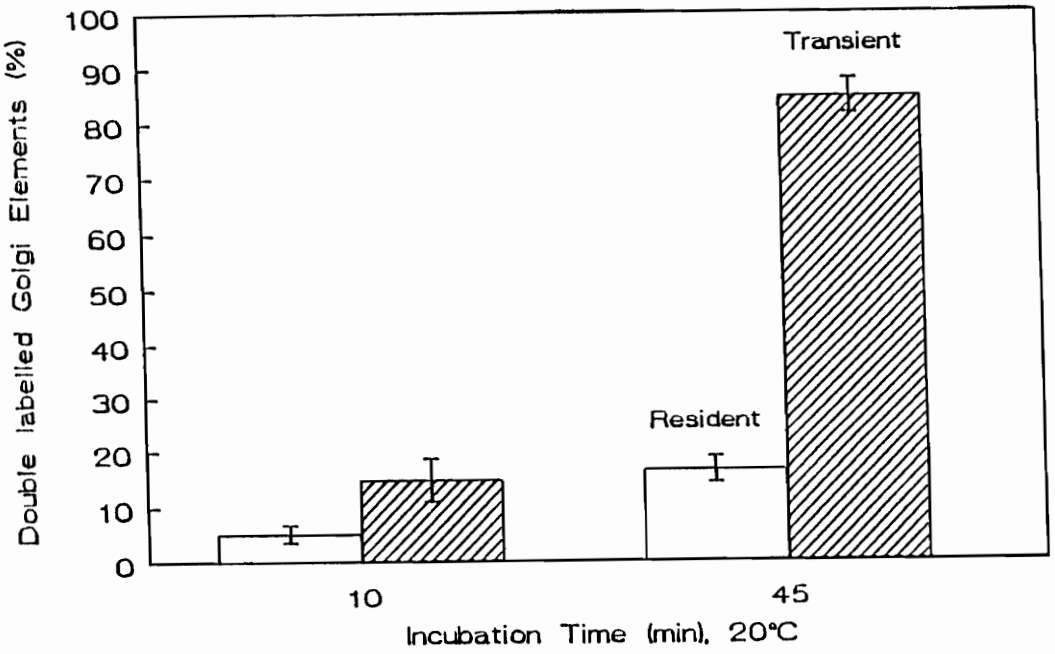


Fig. 5-11. Comparative kinetics of resident and transient Golgi membrane protein co-localization at 20° C. NRK and 15B cells were fused in the presence of cycloheximide and triple immunofluorescently stained for $GIMP_c$, Golgp125 and VSV-G protein as in Fig. 5-10. Micrographs were taken and scored the percentage of the Golgi apparatus doubly positive for $GIMP_c$-Golgp125, or $GIMP_c$-VSV G protein.

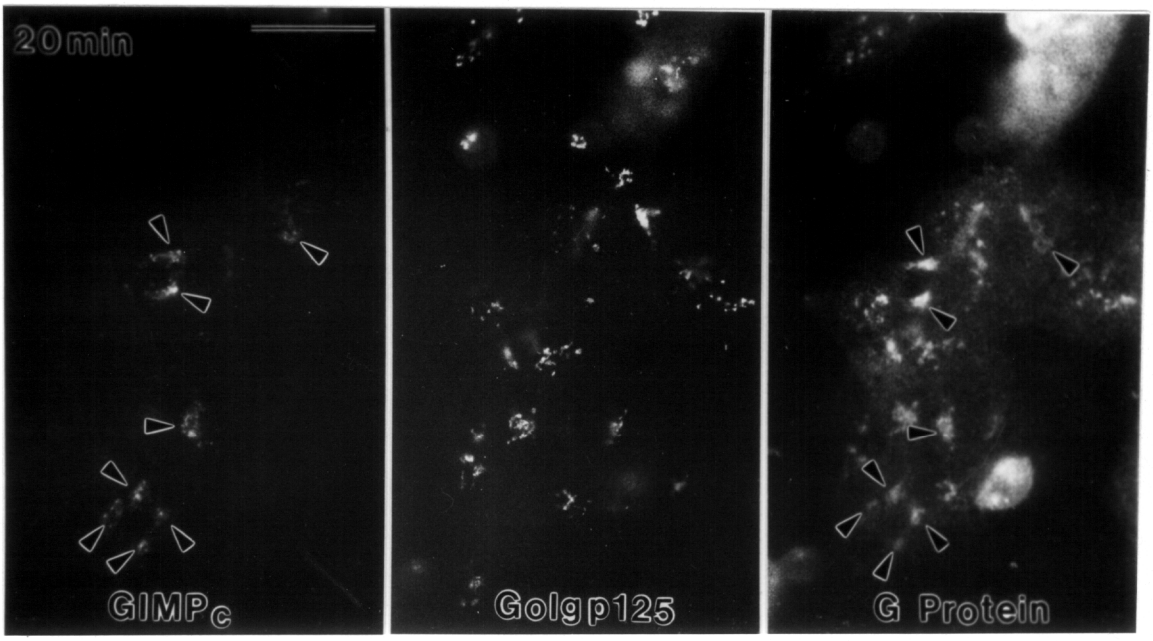
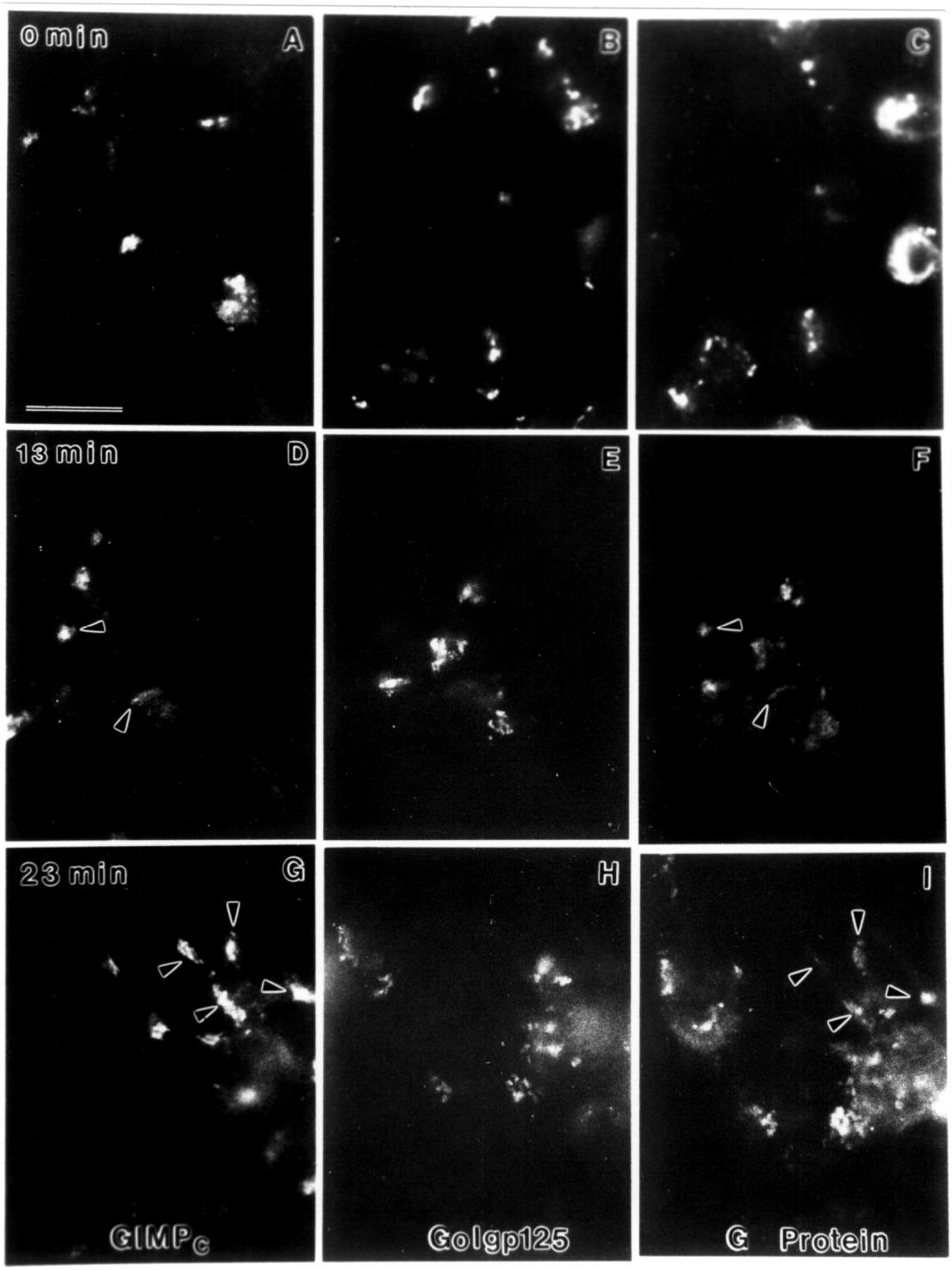


Fig. 5-12. Triple immunofluorescent labeling of $GIMP_c$, Golgp125 and VSV-G protein in NRK-15B cells. NRK and 15B cells were fused in the presence of cycloheximide 2.5 h after the infection of 15B cells with wild type VSV. Cells were incubated at 37° C for 20 min after the fusion protocol and fixed. Cells were triple stained for $GIMP_c$, Golgp125 and VSV-G as described in Fig. 5-10. Micrographs were taken in the same focal plane but in different fluorescent channels. Frame A, fluorescein channel, showing the $GIMP_c$ staining pattern; frame B, Texas Red channel, showing the Golgp125 staining pattern; frame C, coumarin blue channel, showing the VSV-G protein staining pattern. Arrowheads point to Golgi apparatus positive for both $GIMP_c$ and VSV-G protein. Magnification = 916 X, Bar = 20 μm.

Fig. 5-13. Triple immunofluorescent localization of $GIMP_c$, Golgp125 and tsO45-G protein in NRK-15B fused cells. 15B cells were infected with tsO45 VSV as described in materials and methods. Cycloheximide was added to the culture 30 min before cell fusion and the syncytia were incubated in the presence of cycloheximide. NRk and the infected cells were fused and cells were incubated at 39.5° C. Syncytia were fixed at different time points and stained for $GIMP_c$, Golgp125 and tsO45-G protein as described in Fig. 5-10. $GIMP_c$ staining pattern, frame A,D,G; Golgp125, B,E,H; and tsO45-G protein, C,F,G. For each time point (A,B,C; D,E,F; G,H,I), micrographs were taken in the same focal plane but in different fluorescent channels. Arrowheads point to the Golgi apparatus positive for both $GIMP_c$ and tsO45-G protein. Magnification = 853 X, Bar = 20 μm.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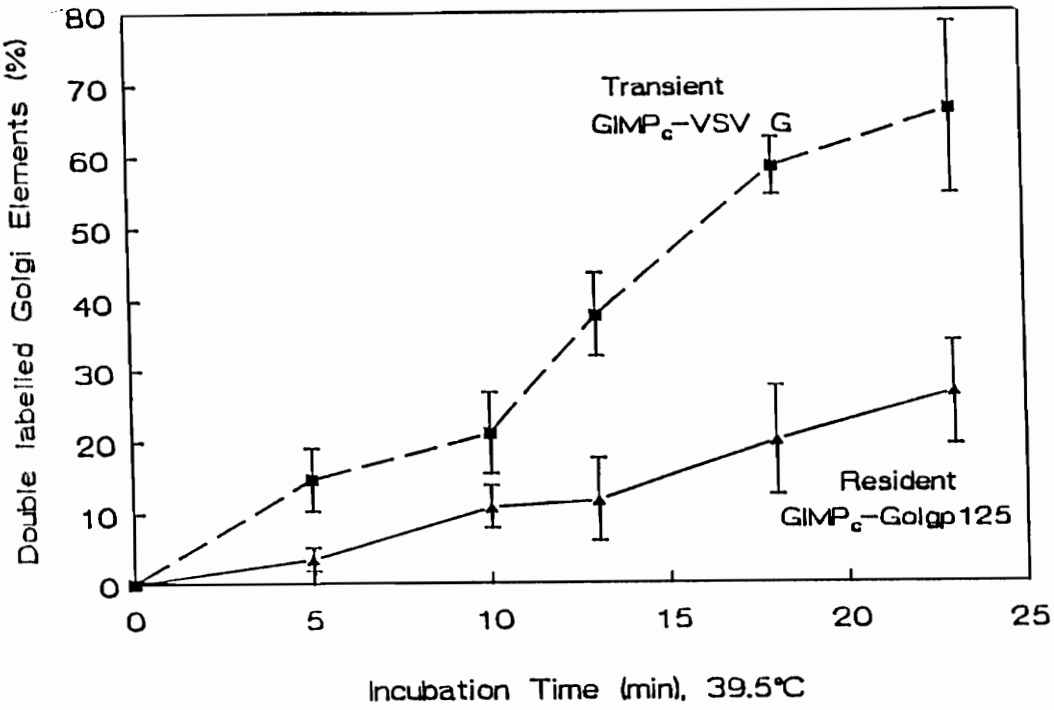

Fig. 5-14. Comparative kinetics of resident and transient Golgi membrane protein co-localization at 39.5 °C. 15B cells were infected with tsO45, treated with cycloheximide, and fused with NRK cells as described in Fig. 5-13. Micrographs were taken and printed for scoring the percentage of the Golgi apparatus positive for GIMP$_c$-Golgp125, or GIMP$_c$-tsO45 G protein as described in Fig. 5-11.

Chapter 6. General Discussion

6.1. Lysosomal content and membrane protein intermixing in fused cells

One of the major outcomes of my thesis work was to show that lysosomal contents and membrane proteins rapidly intermixed in fused cells. It is likely that the other multi-copy organelles, like the peroxisome and the mitochrondrion, may also be as dynamic as the lysosome, i.e., different members within a multi-copy organelle are intercommunicating.

Two different assays for lysosomal content exchange were both conducted by invertase-sucrose pairing, a enzyme-substrate pairing method. One population of lysosomes was loaded with invertase and the other population of lysosomes was loaded with sucrose by incubating two groups of cells in either invertase or sucrose-containing media. The two populations of lysosomes were then brought together into the same cytoplasm by cell fusion. In the first assay, one of the parent cell lines was CHO which developed sucrosomes when incubated with 0.03 M sucrose for 20 h. Sucrosomes are swollen, big, easy-visualized lysosomes. The lysosomal content intermixing was then analyzed by phase-constrast microscopy by observing the decrease in number of sucrosomes in fused cells. This invertase-lysosomes/sucrosomes assay has been reported previously (Ferris et al., 1987). There are some disadvantages to this assay. Firstly, one of the parent cell lines used in the cell fusion in limited to that can develop sucrosomes because not all cell lines can develop sucrosomes when incubated with sucrose. Secondly, the sensitivity of this assay is limited. After cell fusion, sucrosomes become smaller and then disappear. However, only the disappearance of sucrosomes will not fully represent when the content

intermixing starts. Accordingly, a second assay which is more sensitive was developed in my study. In this second assay, a trace amount of [^{14}C]-sucrose was used to feed the donor cells. The content intermixing was then monitored by the amount of [^{14}C]-glucose and fructose released to the culture medium. Glucose and fructose are the degradation products of [^{14}]-sucrose by invertase digestion. The second assay was much more sensitive than the first one. With the use of this sensitive invertase-lysosome/[^{14}C]-sucrose-lysosome assay, lysosomal contents were shown to intermix rapidly with no lag period. The intermixing was inhibited greatly by ATP inhibitors and partially by cytochalasin D. Cytochalasin D is both a cytofilament disrupter and a glucose-uptake inhibitor. The partial inhibition of content intermixing by cytochalasin D may well be due to the energy (ATP) depleting effect.

The assay for lysosomal membrane protein intermixing was achieved by analyzing the distribution of lysosomal membrane proteins recognized by species-specific monoclonal antibodies. It was first shown by the transfer of lysosomal membrane protein LAMP-2 from mouse lysosomes to hamster sucrosomes at the immunofluorescent level. With the use of the sucrosome, a big, easy visualized vacuoles under light microscope, the transfer of LAMP-2 was observed clearly with no z-dimensional ambiguity. The lysosomal membrane protein intermixing was also observed by the co-localization of the two lysosomal membrane proteins, rat LIMP-I and mouse mLAMP-1 in NRK-3T3 syncytia, shown by double immunofluorescent staining at light microscopy level and by double immunogold labeling of cryosections at the electron microscopy level. The lysosomal intermixing was rapid, with a $t_{1/2}$ = 30 min, which is consistent with the lysosomal content intermixing kinetics.

Lysosomal content or membrane protein intermixing has never been shown before. In fact in *in vitro* experiments, late endosomes or lysosomes were shown not

be able to intermix, although early endosomes are able to intermix (Gruenberg et al., 1989; Gruenberg and Howell, 1986; Woodman and Warren, 1988). This probably has something to do with the lack of intact microtubules *in vitro*. Experiments show that disruptions of microtubules stop the transport of materials from endosomes to lysosomes (De Brabander et al., 1988; Oka and Weigel, 1983). Lysosomes also have been shown to move on the tracks of microtubules in live cells (Matteoni and Kreis, 1987). My study also shows that nocodazole, a microtubule disrupter, inhibits the lysosomal content and membrane intermixing.

Cell fusion has been used as an experimental approach in these experiments. Cell fusion can be mediated by live virus (chapter 2 and 3), UV-inactivated virus (chapter 4 and 5) or polyethylene glycol (PEG, chapter 4). As far as the experimental results, no difference was observed with different cell fusion methods. Fused cells have been shown to have normal metabolic activities as in single cells, shown by the ability of the fused cells to take up radioactive labeled DNA, RNA and protein substrates (Harris, 1970). In chapter 4, fused cells were also shown to have normal endocytosis compartment size and nucleus/cytosol ratios. All these observations indicate that cell fusion is an useful experimental approach. Lysosomal content and membrane protein intermixing that occurred in the fused cells may well happen in the single cells as well. Microinjection can be used to test this. To study the lysosomal content intermixing in single cells, one population of lysosomes labeled with a fluorescent marker can be isolated and then injected into the cells in which the lysosomes are labeled a with different fluorescent dye. After the injection, occurence of lysosomal content intermix could be observed by fluorescent light microscopy.

The lysosomal content and membrane intermixing may have several implications in the study of the lysosome. Firstly, the newly synthesized lysosomal content

enzymes and membrane proteins will be distributed through out the lysosomal population. Secondly, the lysosomal function, hydrolysis of macromolecules, will be carried on by intermixing the lysosomal contents, acid hydrolases and substrates, between different members of the lysosome population.

6.2. Comparative behavior of the PLC and the lysosome in fused cells

The PLC and the lysosome are two functionally interrelated organelles. They share many common features and are both involved in the endocytosis pathway (Griffiths et al., 1988). However, they were shown to behave differently in cell fusion experiments. In fused cells, the initially separate PLC units congregated to form an extended PLC complex associated with the nucleus cluster. Lysosomes, on the other hand, remained small, punctate and scattered throughout the cytoplasm despite the fact that the lysosomal contents and membrane intermixed. These different behaviors of the PLC and the lysosome suggest that there may be different pathways for membrane protein and content transport for different organelles.

The extended PLC complex in 3 h old syncytia was shown to function like a single organelle. It is likely that PLC membrane proteins also intermixed by then. This can readily be tested by using species-specific monoclonal antibodies to different species MPRs.

6.3. The mechanisms of lysosomal content and membrane protein intermixing

There are several possible mechanisms for the lysosomal content and membrane protein exchange. One is by direct contact between different copies of lysosomes. Lysosomes may be connected together by a thin tubular network. Actually, thin tubular lysosomes have been shown before (Swanson et al., 1987; Ferris et al., 1987).

Preliminary experiments indicated that the different amounts of pre-existing tubular lysosomes in the cells did not result in different rates of lysosomal intermixing (chapter 2). Further experiments are necessary to have a concrete conclusion. One experiment may be to induce the tubular lysosomes by PMA treatment. The rate of lysosomal content intermixing with or without the presence of PMA may then be compared by the sensitive invertase-lysosome/[^{14}C]-sucrose-lysosome assay.

The second possible mechanism for lysosomal intermixing is by small vesicles budding from and then fusing with lysosomes. Recent studies have shown that many interorganelle protein transports are mediated by small vesicles. For example, proteins transported from the endoplamic reticulum to the Golgi apparatus and protein transported between Golgi stack cisternae, are transported via small vesicles budding from the donor and fusing to the recipient (Rothman et al., 1984a,b; Beckers et al., 1989). However, there is no direct evidence that lysosome-lysosome communication occurs via small vesicles.

A third possible mechanism is that lysosomal exchange is mediated by the PLC. This proposal is based on the facts that the PLC and the lysosome are two interrelated organelles. They share many common membrane proteins and contents. Also, when the cells were endocytoticly labeled with colloidal gold particles, 50-50 of the gold particles distributed in the PLC and the lysosome. This indicates that there may be an equilibrium between these two organelles. Since the PLC units congregated together in the fused cells, and probably had their content and membrane protein intermixed, it is possible that the lysosomal content and membrane protein get exchanged by commuting between lysosomes and the mixed PLC.

Lysosomal intermixing by direct lysosome-lysosome contact also is possible. In the nocodazole treated cells, PLC units were dispersed by nocodazole; at the same

time lysosomal intermixing was inhibited but not completely aborted. In addition, when the fused cells were treated with low pH medium, lysosomes dispersed and moved outward from the center of the cells where the PLC is usually located. Under these low pH conditions, lysosomal membrane protein intermixing still occurred as at neutral pH condition. Therefore, at this stage, any conclusion about the role of the PLC in lysosomal intermixing will be premature and further experiments are needed. The fourth possible mechanism for lysosomal intermixing is by lysosomal fusion and fission, which I think is the most likely mechanism. There are several experimental results which support this possible mechanism. Firstly, with time-lapse video recording, lysosomes were observed to move rapidly in the cytosol. The movement usually is short in distance. This movement results in many chances for lysosomes to bump into each other. Once these two lysosomes bump together, they stop momentarily and then separate and move apart (see chapter 4 and appendix 2). Lysosomal content and membrane protein exchange may occur during the brief moment when two lysosomes meet. Since the lysosomal movement is short in distance, the lysosomal content and membrane protein intermixing may be extended by relay. This relay may result in the uneven spread of lysosomal contents and membrane proteins. As a matter of fact, this is what has been observed in the fused cells. One experiment to test this fusion/fission mechanism is to label lysosomes with two differnt fluorescent dyes in two groups of cells respectively and then fuse these cells together. The movement of lysosomes are then recorded by time-lapse video connected to the fluorescent microscope. If two lysosomes with different colors meet each other and then gain each other's fluorescent dyes, that might prove that lysosomal exchange occurs by fusion and fission. For this experiment, a low light, highly sensitive fluorescent camera may be required.

6.4. Golgi resident and transient membrane proteins transport in fused cells

With the cell fusion approach, the comparative kinetics of resident and transient membrane protein intermixing between neighboring Golgi apparatus was investigated. Experimental results show that transient Golgi membrane proteins, VSV-G or tsO-G protein, was transported from the hamster to the rat Golgi units much faster than the intermixing of resident Golgi membrane protein, $GIMP_c$ or Golgp125. The resident Golgi membrane protein intermixing was accompanied by the congregation of Golgi units; the transient Golgi membrane protein was transported between the separate Golgi units. When Golgi units were congregated together, it is likely that interconnections were formed between the Golgi units. Experiments done by other researchers have shown that *trans*-Golgi cisternae contract and extend, breaking and forming connections with the adjacent Golgi tubule (Cooper et al., 1990). In cell fusion experiments, tubular connections were seen within the congregated Golgi complex 30 min to 1 h after cell fusion (Xiao and Storrie, 1991). These results indicate that there are two different pathways for the Golgi membrane protein transport; one is a rapid, selective, and vesicle-mediated pathway for Golgi transient membrane proteins and the other is a slow, lateral diffusion pathway for resident Golgi membrane proteins.

The transient Golgi membrane protein transport experiments indicated that vesicle-mediated transport may also be the pathway for protein transport between the Golgi cisternae in single cells. The resident membrane protein transport results suggest that the Golgi membrane protein is mobile. Further experiments will be needed to observe the actual resident membrane protein mobility along the Golgi cisternae. The mobility of a resident membrane protein can be detected by a laser

bleaching experiment. By microinjection of antibodies against the cytoplasmic portion of the protein, the selected resident Golgi membrane protein can be fluorescently labeled inside the live cells. To see if the membrane protein diffuses along the tubular elements, a fraction of the element can be bleached by applying a laser beam. If the labeled protein reappears in that fraction rapidly, it will indicate that the membrane protein do diffuse along with the tubular connection. Confocal fluorescent microscopy may be required to have a clear observation in this experiment.

6.5. Comparison between resident protein intermixing within lysosome-lysosome versus Golgi-Golgi/PLC-PLC

Both the Golgi apparatus and the PLC are single-copy, perinuclear organelles. In fused cells, the behavior of PLC and the Golgi apparatus are very similar. They both congregate together in association with the clustered nuclei. Therefore, the mechanisms for resident membrane protein intermixing for PLC-PLC and Golgi-Golgi can be considered the same.

In contrast, for lysosomes, the resident membrane protein occurred without a change of lysosome size and distribution, i. e., lysosomes remained small, punctate and dispersed throughout the cytosol. Therefore, the mechanism for lysosomal membrane protein intermixing and the mechanism for Golgi/PLC membrane protein intermixing are different. For lysosomes, protein intermixing is likely occurred by a fusion/fission mechanism; while for Golgi/PLC, it likely occurred by lateral diffusion along with the conjunctions which are formed between Golgi/PLC units when they congregate together.

6.6. Proposal for the study of biochemical mechanisms of protein intermixing

within lysosomes/Golgi/PLC

To study the biochemical mechanism of the intermixing within a organelle, cell-free or semi-intact cell systems will be necessary. For example, to study if the lysosomal membrane protein intermixing is GTP or calcium dependent, using semi-intact cells would be more appropriate than using intact cells because the GTPgamma S inhibitors or calcium binders will enter the cell directly and, the experimental result will be easily to interpret. Semi-intact cells can be achieved by permeabilizing the cell membrane. One of the methods is to put a piece of filter paper on a monolayer of cultured cell and then take off the filter quickly. Part of the plasma membrane will be torn away with the filter paper and the cells will be permeable.

A cell-free, or *in vitro* system is another approach. Basically, an organelle population can be isolated and purified by cell fractionation. In comparison to semi-intact cells, cell-free system approach has the advantage of giving more direct interpretation of the result. One the other hand, the interpretation is also dependent on the purity and the integrity of the organelle fraction.

Recently, an N-ethylmaleimide-(NEM, a sulphhydryl blocker) sensitive protein (NSF) was reported to be essential to many vesicle transports, including the transfer of vesicles between different regions of the Golgi complex, the transfer of vesicles from the rough endoplamic reticulum to the Golgi apparatus, and the fusion of endosomes (Becker et al, 1989; Diaz et al., 1989; Schatz, 1985). This NSF protein has been characterized and cloned (Wilson et al., 1989). It is a tetramer with subunits of molecular weight of 76 kD, and it is found in both soluble and membrane-bound form. In the presence of ATP, NSF is released from the membrane and becomes soluble (Block et al., 1988). NSF is necessary for the vesicle fusion step but not for the vesicle binding step. Since NSF is required for so many different branches of

protein transport or vesicle fusion, it is likely that NSF is also required for the protein intermixing within an organelle population. To test this possibility, either NEM or NSF antibody could be added to the semi-intact syncytia.

With the use of a semi-intact cell or cell-free system, many other factors can be analyzed to determine whether they are required for the protein intermixing within an organelle population. This will help us to understanding the mechanism of intraorganelle dynamics at the molecular level.

6.7 References

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Appendix 1. Acid cytoplamic pH effects on the shape, size, and distribution of the prelysosomal compartment (PLC) and lysosomes in NRK-3T3 fused cells

A previous report has showed that lysosome distribution, shape and size changed when the culture medium pH was changed from neutral (pH ~7.2) to acid pH (6.9 to pH 6.5) (Heuser, 1989). When cells are cultured in neutral pH medium, lysosomes are usually clustered around the nucleus. When the culture medium pH was shifted to acid (pH 6.9-6.5), lysosomes were observed to move from the center of the cells to the edge of the cells. Lysosomes also became bigger and more rounded (Heuser, 1989). To investigate if the low pH had any effect on the distribution and morphology of the pre-lysosomal compartment (PLC), 3T3 cell cultures were shifted to acid pH medium (pH 6.9 Ringer's acetate medium, see reference) for 15 min. Cells were fixed and immunofluorescently stained with anti MPR-antibody. No change in the PLC distribution, size and shape was observed (data not shown). To investigate if the low pH had any effect on lysosomal membrane protein intermixing, NRK and 3T3 were fused by a UV-inactivated Sindbis virus mediated process (Chapter 4). Cells were then cultured in the pH 6.9 Ringer's solution for 3 h at 37°C in a water-bath. Cells were fixed and immunofluorescently double stained for LIMP I and mLAMP-1. The experimental results showed that lysosomal membrane protein exchange was as extensive as when syncytia were cultured in neutral pH medium (data not shown). However, when the syncytia were cultured in pH 6.9 medium, lysosomes appeared to be bigger and more rounded. Also the lysosomes were located more toward the edge of the syncytia rather clustered around the nuclei. These changes are consistent with the results reported by John Heuser.

Reference:

Heuser, J.: Changes in lysosome shape and distribution correlated with change with cytoplamic pH. J. Cell Biol. **108**, 855-864 (1989).

Appendix 2. Study of lysosomal movement by time-lapse video microscopy.

Studying the changes of lysosomes distribution inside a living cell may help us to find out the mechanism of lysosomal content and membrane exchange. 3T3 cells were chosen in this study because the they are the most spread and flattest cells among the cell lines in the laboratory. 3T3 cells were cultured in a tissue culture dish in which a hole was cut in the middle and a coverslip was mounted on the hole. Mounting a coverslip on the bottom of the tissue culture dish allowed us to observe the fluorescence more clearly at high magnification. High magnification lenses are capable of focusing through only short distances. To fluorescently label the lysosomes in 3T3 cells, 100 μg/ml Texas Red conjugated-dextran was added to the 3T3 culture. Cells were cultured with the dextran overnight and then incubated in dextran-free medium for 2 h as a chase to clear dextran from endosomes. The dish in which 3T3 cells were cultured was transfer from the incubator to the stage of a fluorescent microscope. The culture was maintained at 37°C and in 5% CO_2 environment on a heated stage equipped with a gas manifold. The excitation light for fluorescent microscopy was shuttered on periodically to shine on the sample. This reduced exposure of the cell to the fluorescent light and prevent photobleaching. Every time the fluorescent light was shuttered on, four frames of the image were recorded in the video recorder through a video camera. Lysosome movement was analyzed by playing the VHS format video tape. Under these procedures, the movement of lysosomes was speeded up and was more obvious. The experimental results showed that lysosomes moved rapidly within the cytoplasm. Lysosomes

seemed to move back and forth toward and outward the center of the cell along some tracks. The movements usually were short in distance. There were many cases in which two lysosomes met each other. The meeting was brief, lasting only 3 to 5 seconds, and two the lysosomes separated again. In the presence of ATP inhibitors (50 mM 2-deoxyglucose and 1 mM sodium cyanide) or a microtubule disrupter (5 μg/ml nocodazole), the lysosome movement decreased dramatically, and there was much less lysosome-lysosome contact. The experimental results indicated that lysosomal membrane and content exchange may happen during the brief moment when two lysosomes meet. The exchange may then be extended by passing the lysosomal membrane and content from one lysosome to another lysosome by a "relay" manner. Further experiments are necessary to have a solid conclusion (see chapter 6).

Appendix 3. Detailed protocol for cell fusion mediated with UV-inactivated Sindbis virus.

I. Large scale Sindbis virus preparation

1. Culture BHK-21 cells overnight in 175 cm^2 tissue culture flasks (preferably Nunc) in MEM supplemented with 10% fetal calf serum. Sixty flasks will give about 2 ml of concentrated virus at the end. Plated about 2 X 10^6 of cells in each flask so that cells will be 80-90% confluent the next day.
2. The next day. Wash the cells 2X with PBS containing 0.2% (w/v) BSA. Inoculate the cells with 2.5 ml of virus per flask diluted in Earle's MEM (0.2% BSA, 1% penicillin and streptomycin, 1% Glutamine, and 10 mM HEPES pH7.2). The virus concentration should be about 5 pfu/cell (see below for plaque assay).
3. Incubate the cells with virus for 1 h at 37°C and tilt the flasks once to ensure an even spread of virus.
4. Add 15 ml/flask maintenance medium (EMEM plus 5% trytose phosphate broth) and incubate the cells overnight in the incubator.
5. Check frequently from 20 hours onwards for cytopathic effects. The virus is budded into the medium. The medium is ready to be harvested when cells are vacuolated and rounded up but before they are released into the medium. This is usually 20-24 hours post infection.
6. Place flasks upright in 4°C for 30 min. Pool the medium from the flasks and centrifuge at 10.000 rpm for 30 min at 4°C.
7. Pool the supernatant and concentrate the virus with the Millipore Minicon concentrator with 300-KD cutoff filter. This step will concentrate the virus

about ten times.

8. Centrifuge the retentate over 3 ml cushions of 20% glycerol in PBS in an SW27 rotor at 27,000 rpm for 5 h at 4°C.
9. Resuspend pellets in 0.5 ml of TN (50 mM Tris-HCL pH7.6, 100 mM NaCL) containing 0.2% (w/v) BSA on a rocker in the coldroom overnight. This will provide three to 5 ml of virus. This will concentrate the virus another 40 times.
10. Divide the virus into eppendorf tubes with 10 to 20 μl in each tube, keep them in -80°C freezer.

II. Plaque assay

1. Culture about 5 X 10^5 cells in 60 mm tissue culture dishes. The amount of cells should give half confluence the next day.
2. Infect the cells with virus with various dilutions. Virus is diluted in culture medium supplemented with 2% fetal calf serum. Add 250 μl of virus per dish.
3. Combine equal volumes of 2X culture medium containing 2X of serum (at 37°C) with 2% autoclaved low melt agarose (at 37°C). Overlay the cells with 5 ml agarose/medium mixture.
4. Incubate the cells at 37°C incubator and observe periodically. It usually takes more than 12 h to form plaques.
5. Plate the dishes in cold for a few minutes and pull off the agar.
6. Stained the cells with 0.6% neutral red in complete culture medium for 3 h.
7. Rinse the cells with water and count plaques.

II. UV-inactivation of virus

1. UV-inactivate the virus before they are used for cell fusion. Place a 1 ml eppendorf tubes containing 10 μl virus on a ice bucket. Put the ice bucket under a 40-W germicidal lamp of a laminar flow hood. The distance between the lamp and the tube should be 50 cm. UV-treated for 30 min.
2. Dilute the virus in binding buffer (pH 6.8, see below). Always keep the virus on ice.

III. Fusion buffers

1. Make up MEM medium, do not add sodium bicarbonate.
2. Add HEPES, MES and BSA to final concentrations of 10 mM HEPES, 10 mM MES, and 0.2% BSA.
3. Divide the medium into four parts. Adjust the pH of each batches respectively to pH 5.5 (fusion buffer), pH 6.0 and pH 6.5 (transition buffers), or pH 6.8 (binding buffer).
4. Filter the buffers to sterilize and keep them in a refrigerator.

IV. Cell fusion

1. One day before cell fusion experiments, plate recipient cells on tissue culture dishes. For immunofluorescent staining, the dishes should contain coverslips, preferably 12 mm round coverslips. The amount of cells plated varies from 1 X 10^6 to 3.5 X 10^6 per 60 mm tissue culture dish, depending on the cell line used (3T3, 1.0 X 10^6; NRK or CHO, 1.5 X 10^6; 15B, 3.5 X 10^6). The amount of cell used should give about 70% confluence the next day. To make sure the right amount of cells are there on the next day, several dishes with slightly

different concentration of cells can be set up although only one dish in needed. If cells fusions are done on coverslips, more recipient cells should be plated to have a extensive cell fusion than when cell fusions are done directly on the tissue culture dishes. Cells are slightly more spread on plastic than on glass.

2. On the day of cell fusion, donor cell are added to the recipient cells. Again, the cell numbers depend on cell lines. The amount of donor cells plated should give about equal numbers of donor and recipient cells. Mixed cells are co-culture for at least 2 h to let the donor cell attach.
3. Rinse the cells with chilled binding buffer (pH 6.8) 2 X and then add the diluted UV-inactivated virus to the cells. Virus dilutions used depend on different cell pairings and can be decided experimentally. Usually, homologous cell fusion requires less concentrated virus than heterologous cell fusion. Also, with the same cell pairing, less virus is required for a cell fusion done directly on the tissue culture dishes than on coverslips. When coverslips are used, dry the bottom of each coverslip, put it into a chilled dish before adding virus. About 15 μl of virus is enough for each coverslip and 300 μl for each 60 mm tissue culture dish. Incubate the virus with cells on ice for 30 min.
4. Slowly add room temperature fusion buffer (pH 5.5) to the cultures. Three minutes later, rinse with room temperature pH 6.0 and pH 6.5 transition buffers. Allow about 1 min for each rinse. Allow fusion to proceed by incubating the cells at 37°C with culture medium supplemented with 5% fetal calf serum.

Appendix 4. Detail protocol for immunogold labeling of cryosection

I. preparation of sample in frozen blocks

1. Put the tissue culture dish in which cells are grown on ice and keep it on ice for the following procedures.
2. Rinse the cells 2X with ice cold PBS.
3. Add 1 ml of diluted, ice cold Proteinase K solution to the cells. Proteinase K is prepared as a stock solution at 10 mg/ml in PBS and kept in freezer and diluted in ice cold PBS before used, 1:100 for fused cells, 1:200 for NRK or 3T3, 1:300 for BHK. Wait about 2 min, cells should come off the dishes easily, otherwise, increase the Protein K concentration. But do not wait long. Unlike trypsin treatment, proteinase K dose not cause cells to round up. Collect the cells gently into a small tube with a Pasteur glass pipette whose thin end is broken off.
4. Add 200 μl of fixative (either 8% formaldehyde or 1% glutaraldehyde in 250 mM HEPES pH7.4).
5. Spin down the cells for 1 min at 3000 rpm (1000-1500g).
6. Take off the supernatant without disturbing the pellet and add fresh fixative. Leave the cells at room temperature. 5-30 min later, spin the cells at 13,000 rpm for about 1-5 min.
7. Place the specimen in 2.1-2.3 M sucrose in PBS for 30 min.
8. Trim the specimen if it is too big and then mount it on a copper stub under an binocular microscope. Be careful not to allow the specimen to dry out.
9. Plunge the cooper stub immediately into liquid nitrogen with shaking. Wait until

the bubbling of nitrogen gas stops. Put the stub into a labeled small eppendorf tube with the top of the stub facing down and store the stub in a liquid nitrogen container until ready to used.

II. Cryo sectioning with a cryo-ultramicrotome

1. Precool the microtome with liquid nitrogen to about -100°C before putting the specimen in. Put sample in chamber and allow 5-10 min for temperature equilibrating before starting to cut.
2. Thin sections are grouped together with a eyelash attached to a stick. Sections are picked up by a copper or platinum loop containing 2.1 M sucrose in PBS. Attach the sections on a grid by touching the loop on the surface of the grid. The grid is 100 mesh, hexagons which has a carbon-coated formvar film. Invert the grid and pace it on PBS solution. The grids will float on the surface of the PBS solution. Collect more than one grid for each staining.

III. Immunogold labeling

The following procedures are performed on a piece of paraform at room temperature. Grids are transferred between different solutions which are plated on the paraform in drops. The transfer can be either by a tweezers or a 3 mm diameter loop.

1. Incubate the sections with 10% FCS in PBS containing 0.02 M glycine for 10 min.
2. Incubate the sections with first antibody for 30 min. Antibody is diluted in PBS containing 5% FCS and 0.02 M glycine. The dilution varies from 1:10 to 1:2000, and usually it is ten times more concentrated than that used in immunofluorescent staining. As little as 3 μl may be used for each grid.

3. Rinse 4X in drops of PBS + 0.02 M glycine for a total 15 min.
4. Incubate with protein A-conjugated colloidal gold for 30 min.
5. Rinse 6X with PBS with a total of 20 min.
6. For double staining, incubate with free protein A (0.2 mg/ml) for 20 min. Repeat steps 2 - 5 for the next set of first and second antibody. The size of the gold particles should be different from the previous one.
7. Rinse 4X in distilled water for a total of 10 min.
8. Rinsed 2X with distilled water for a total of 1 min.
9. Incubate on ice with mixture of methyl cellulose (9 part) and 3% aqueous uranyl acetate (1 part). Pick up the grids with a 3-3.5 mm loop and air dry the grid in the loop. Take the grid off the loop with a sharp tweezers by breaking the film between the edge of the grid and the loop. The grid is ready for electron microscopy examination.

Appendix 5. Localization of a 76 kD endoplasmic reticulum membrane protein by immunogold electron microscopy

Monoclonal antibody H69 was raised against plasma membrane-enriched fractions of mouse NIH/3T3 cells (done by Daryll E. Warder in Thomas August laboratory, John Hopkins, Baltimore, MD, also see Warder et al., in preparation). The H69 antigen shows a molecular weight of 76 kD by SDS-PAGE of immunoprecipitates and was an integral membrane protein. When NIH/3T3 cells were immunofluorescently stained with H69, they showed a endoplasmic reticulum-like staining pattern (Warder et al., in preparation).

Electron microscopy of immunogold stained cryosections was used to further characterize the subcellular localization of H69 antigen. NIH/3T3 cells were cryosectioned and then stained with H69 antibody and 9 nm protein A-gold particles (see appendix 4 for detailed procedures). The electron micrographs showed that this 76 kD glycoprotein was located mainly in the endoplasmic reticulum, with some in the nuclear envelope and plasma membrane (fig. 1). The nuclear envelope and the endoplasmic reticulum are co-existance. Table 1 shows quantitation of the overall distribution of H69 antigen throughout the cytoplasm. Over 60% of the gold particles were located in the endoplasmic reticulum, indicating a high concentration per unit volume of H69 in the endoplasmic reticulum. The H69 antigen may be considered a endoplamic reticulum membrane antigen.

Reference: Warder, D. E., Y. Deng, B. Storrie and J. T. August: A Glycoprotein localized in the endoplasmic reticulum and developmentally regulated in mouse F9

cells, in preparation, 1991.

Table 1. Distribution of H69 antigen in cryo-sections of NIH/3T3 cells.

Location	Number of Gold Particles	% of Total Gold Particles
Endoplasmic Reticulum	145	57
Nuclear Envelope	5	2
Nucleus	27	11
Plasma membrane	7	3
Mitochondria	7	3
Cytosol	63	25
Golgi Apparatus	0	0

NIH/3T3 cells were cryo-immunogold labeled with H69 and 9 nm protein A-gold particles. Totally 253 gold particles were scored.

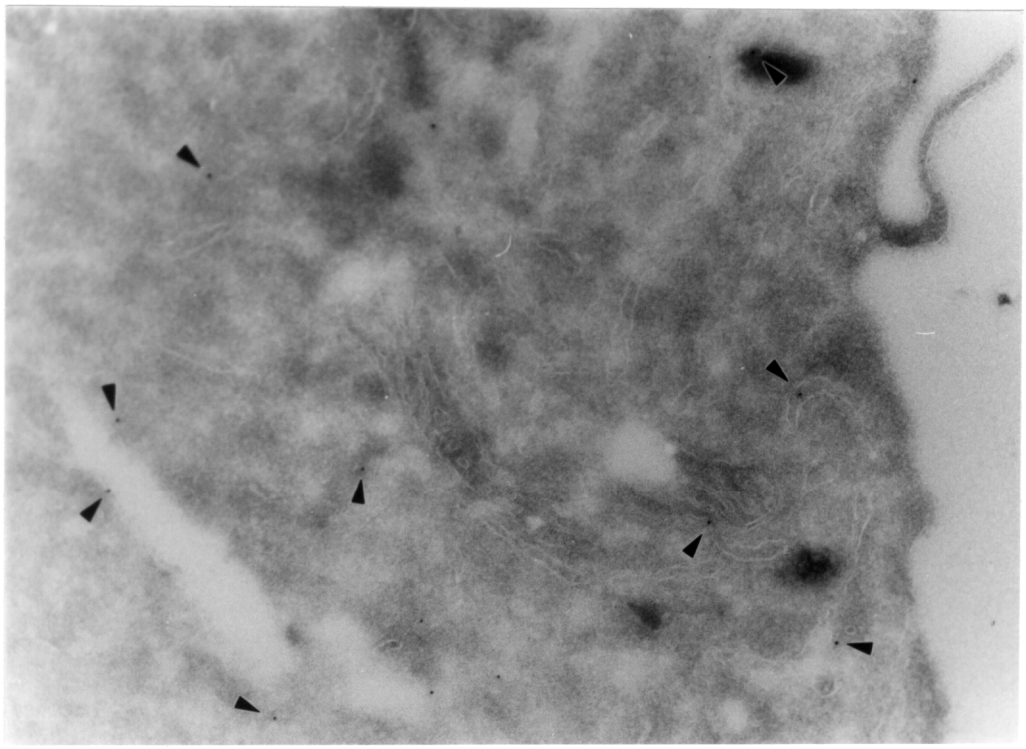

Fig. 1. Cryo-immunogold labeling of NIH/3T3 cells with H69 antibody. NIH/3T3 cells were fixed and sectioned, and labeled with H69 supernatant, rabbit anti rat IgG and 9 nm protein A-gold particles. Arrows point to gold particles associated with endoplasmic reticulum. Magnification = 12,000 X.

Appendix 6. Characterization of radioactive products released in the culture medium by paper chromatography in invertase/[^{14}C]sucrose cell fusion experiments

Cohn and Ehrenreich have reported that in when macrophages were incubated with sucrose, lysosomes were swollen into sucrosomes. When invertase was added to the sucrosome-containing cells, sucrosomes disappeared and fructose and glucose were found to released into the culture medium (Cohn and Ehrenreich, 1968). In my study, sucrosomes disappeared rapidly when sucrosome-containing cells were fused with invertase-containing cells (see chapter 3). To investigate whether the sucrose was also digested by invertase into fructose and glucose, paper chromatography was performed. CHO cells were incubated with 15 μC/ml sucrose overnight and 3T3 cells were incubated with 0.5 mg/ml invertase overnight. The CHO and 3T3 cells were fused together on 30 mm tissue culture dishes and fused cells were grown in 1 ml 50% F12, 50% DMEM plus 2% fetal calf serum. At various time points post cell fusion, the medium (1 ml) was lyophilized. Samples were dissolved in 0.1 ml water and then applied to 1.5" X 20" Whatman No. 1 paper and paper chromatography was run with a solvent of n-butanol:pyridine:water (3:2:1.5). Papers were dried, cut into 2 cm pieces and the CPMs of the pieces were counted with a scintillation counter. Non-radioactive sucrose, fructose and glucose were run as controls and were localized by silver nitrate. The experimental results showed the [^{14}C]-compounds in the medium were not sucrose. No peak co-localized with the standard sucrose. As shown in Fig. 1, the sample migrated rapidly and did not correspond to sucrose or fructose and sucrose. More work will be required to characterize the actual com-

pounds carrying the ^{14}C label. Cohn and Ehrenreich have reported that the only de tected sucrose products seen after invertase entered into macrophage sucrosomes were glucose and fructose (Cohn and Ehrenreich, 1968).

Reference: Cohn, Z. A. and B. A. Ehrenreich (1968). The uptake, storage, and intracellular hydrolysis of carbohydrates by macrophages. J. Exp. Med., **129**, 201-225.

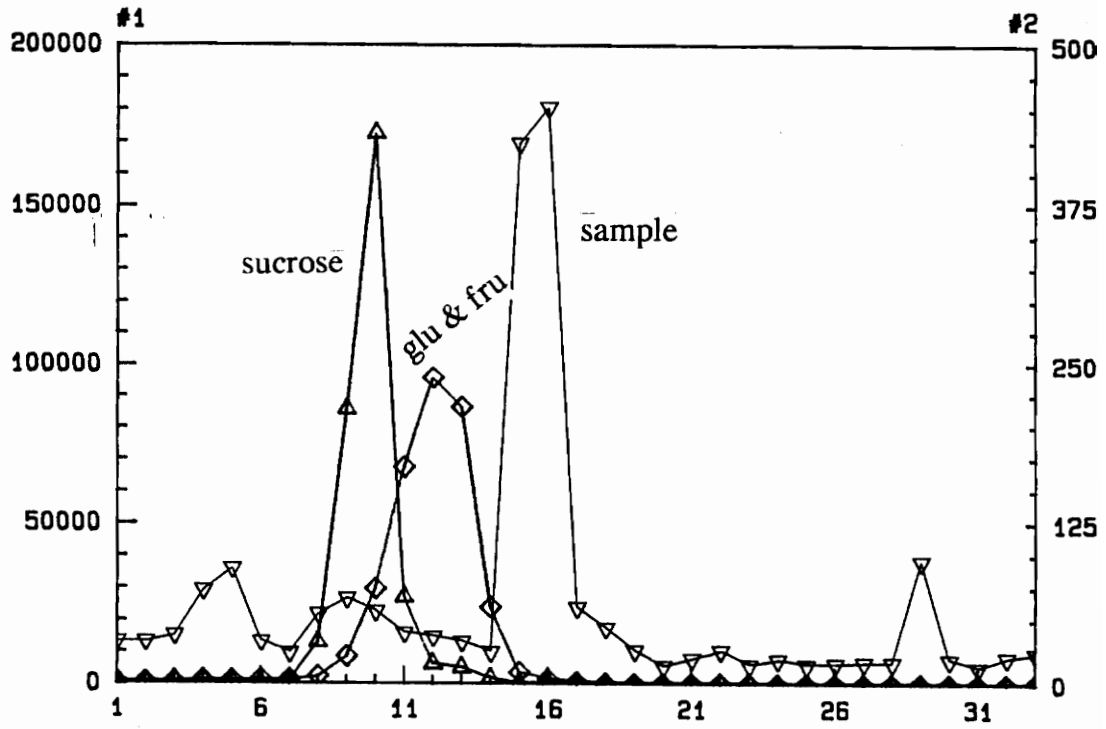

Fig. 1. Paper chromatography of ^{14}C-products from culture medium in invertase/[^{14}C]-sucrose cell fusion experiments. Experimental procedures were described above.

Vita

The author was born in Guangzhou, China on December the fourth, 1963. She received a bachelor degree in Biology from Zhongshan University in 1985. In the same year, she joined the CUSBEA (China and the United States Biochemistry Examination and Application) program. After one year intensive English training at Guangzhou English Language Center, she came to the United States and enrolled in the Ph. D program in the Department of Biochemistry and Nutrition at Virginia Polytechnic Institute and State University.