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Jayasri Das Sarma, Cecilia W. Lo & Michael Koval

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Cx43/β-Gal Inhibits Cx43 Transport in the Golgi Apparatus

JAYASRI DAS SARMA

University of Pennsylvania School of Medicine, Institute for Environmental Medicine and Department of Physiology, Philadelphia, PA 19104

CECILIA W. LO

University of Pennsylvania, Department of Biology, Philadelphia, PA 19104

MICHAEL KOVAL

University of Pennsylvania School of Medicine, Institute for Environmental Medicine and Department of Physiology, Philadelphia, PA 19104

A connexin construct consisting of bacterial β -galactosidase fused to the C-terminus of connexin43 (Cx43/ β -gal) was used to examine Cx43 assembly in NIH 3T3 cells. Cx43/ β -gal is retained in a perinuclear compartment and inhibits Cx43 transport to the cell surface. The intracellular connexin pool trapped by Cx43/ β -gal was retained in a compartment that co-localized with a medial Golgi apparatus marker by immunofluorescence microscopy and that was readily disassembled by treatment with brefeldin A. Further analysis by sucrose gradient fractionation showed that Cx43 and Cx43/ β -gal were assembled into a sub-hexameric complex, and that Cx43/ β -gal expression also inhibited Cx43 assembly into hemichannels. While this is consistent with Cx43 hemichannel assembly in the trans Golgi network (TGN), these data also suggest that the dominant negative effect of Cx43/ β -gal on Cx43 trafficking may reflect a putative sub-hexameric assembly intermediate formed in the Golgi apparatus.

Keywords Gap junction, hemichannel, membrane traffic, protein assembly

INTRODUCTION

Newly synthesized connexins are first assembled into a hexameric hemichannel in an intracellular compartment, followed by transport to the plasma membrane. While some key steps in connexin assembly have been outlined, the intracellular location of connexin assembly into hemichannels remains controversial, with evidence supporting both the endoplasmic reticulum and trans Golgi network (TGN) as assembly sites (reviewed in Yeager et al. (1998)). This situation is complicated by the notion that different connexins may be assembled in different intracellular compartments. Also, to date, discrete intermediates in gap junction hemichannel assembly pathways have not been identified.

NIH-3T3 cells expressing a dominant negative connexin, $Cx43/\beta$ -gal, accumulate Cx43 in the perinuclear region of the cell and show decreased Cx43 transport to the plasma membrane (Das Sarma et al.

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Address correspondence to Michael Koval, University of Pennsylvania School of Medicine, Department of Physiology, B-400 Richards Building/6085, 3700 Hamilton Walk, Philadelphia, PA 19104. E-mail: mkoval@mail.med.upenn.edu

2001; Sullivan and Lo 1995). Since the morphology of the intracellular compartment where Cx43 and Cx43/ β -gal were retained was suggestive of an aspect of the Golgi apparatus or TGN, we decided to further characterize the dominant negative effect of Cx43/ β -gal on Cx43 to determine whether this effect might shed insight into trafficking and assembly of native Cx43.

MATERIALS AND METHODS

Rabbit anti-Cx43 antisera was generated using a 6his-tagged C-terminal tail constructs as previously described (Civitelli et al. 1993). Rhodamineconjugated, FITC-conjugated and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Roche Molecular Biochemicals (Indianapolis, IN). Triton X-100 was from Roche Molecular Biochemicals. Tissue culture reagents were from Life Technologies Inc/GIBCO BRL (Rockville, MD). Unless otherwise specified, all other reagents were from Sigma. The stably transfected NIH 3T3 cells used in this study were previously generated by transfection of expression vectors that encoded for either β -galactosidase (β -gal) or a connexin43/ β galactosidase fusion protein (Cx43/ β -gal) (Sullivan and Lo 1995).

Sucrose gradient fractionation, immunoblot and immunofluorescence were done as described (Koval et al. 1997). To preferentially label the TGN, we used a fluorescent lipid analogue, C₆-NBD-Cer (Pagano et al. 1989). Cells on glass coverslips were washed with PBS, fixed using 4% paraformaldehyde at room temperature for 10 min, washed with PBS, cooled to 4° C, then incubated with 5 pM C₆-NBD-Cer complexed to 0.34 mg/ml BSA. Excess C₆-NBD-Cer was then removed by back exchange using 4×10 min washes at 4°C with PBS containing 3.4 mg/ml defatted BSA and the cells were washed in PBS. The coverslips were then mounted in a microscope stage chamber (Harvard Apparatus, South Natick, MA), covered with PBS and imaged by fluorescence microscopy.

RESULTS AND DISCUSSION

As shown in Figure 1a, NIH-3T3 cells expressing Cx43/ β -gal (43 β 2 cells) show Cx43 enriched in the perinuclear region of the cell and depleted from the plasma membrane. The perinuclear distribution co-localizes with a medial Golgi marker, MG-160 (Das Sarma et al. 2001; Gonatas et al. 1989), consistent with Cx43/ β -gal accumulation in one or more aspects of the Golgi apparatus. Furthermore, when 43 β 2 cells were incubated with brefeldin A for 30 min prior to immunolabeling to stimulate the disassembly of the cis and medial Golgi apparatus stacks into the ER, the bright perinuclear



FIG. 1. Cx43/ β -gal inhibits Cx43 transport and assembly. a. 43 β 2 cells were fixed, permeabilized and immunolabeled for Cx43. Note the prominent immunofluorescence in the perinuclear region of the cell (arrowheads). Bar, 10 μ m. b, c. 43 β 2 cells were treated with BFA for 30 min and then either fixed, permeabilized and immunolabeled for Cx43 (b) or treated with C_6 -NBD-Cer to label the TGN (c). The perinuclear accumulation of $Cx43/\beta$ -gal was dispersed to the periphery of the cell by BFA, indicating it was in an aspect of the Golgi apparatus prior to the TGN. This is in contrast to the effect of BFA on the TGN, which causes it to condense in the region of the centriole (arrows). d. Assembly of native Cx43 into hemichannels (Hex) and sub-hexameric intermediates (Sub-Hex) by control 3T3 cells in either the absence (grey bars) or presence (black bars) of BFA or by $43\beta 2$ cells (white bars) was analyzed by sucrose gradient fractionation and calculated as a percent of the total intracellular Cx43 pool (Triton X-100 soluble). Values reflect the mean \pm standard error for control cells (n = 3), or mean \pm range for the other samples (n = 2).

fluorescence disappeared, indicating that $Cx43/\beta$ gal relocalized to the ER (Figure 1b). This was in contrast to the expected pattern if Cx43 and Cx43/ β gal were localized to the TGN, since this compartment would condense to the region of the centriole in BFA-treated cells (Koval et al. 1997). We confirmed that a 30 min treatment of 43 β 2 cells with BFA caused the TGN to condense, using C₆-NBD-Cer to preferentially label the TGN (Figure 1c). Taken together, these results suggest that most of the intracellular Cx43/ β -gal pool was localized to secretory compartments after the ER and prior to the TGN in 43 β 2 cells.

Our finding that the intracellular pool of $Cx43/\beta$ gal was sensitive to BFA was surprising, since $Cx43/\beta$ -gal interfered with Cx43 transport and the TGN has been implicated as the site for Cx43 assembly in other cell types (Koval et al. 1997; Musil and Goodenough 1993). For instance, Musil and Goodenough used an extensive panel of inhibitors, including BFA, CCCP, 15°C temperature block and CHO cells with a temperature sensitive block in transport to show that assembly of Cx43 into hexamers required transport to the TGN (Musil and Goodenough 1993). In each case, they only detected monomeric Cx43 when transport to the TGN was inhibited. Monensin treatment also caused a 3-4 fold reduction in Cx43 oligomerization in ROS cells, again consistent with the TGN as the site for Cx43 hemichannel assembly (Koval et al. 1997). These data are most consistent with a model for Cx43 assembly where monomers are transported to the TGN, where they are then assembled into oligomeric complexes.

Given this apparent contradiction, we used sucrose gradient fractionation analysis of control and $43\beta2$ cells solubilized in Triton X-100 to determine the oligomeric state of Cx43 and Cx43/ β -gal complexes (Figure 1d). Assembly states for Cx43 were resolved into monomer, sub-hexameric intermediates and hexamer fractions. In control cells, 14.8 ± 4.6% (mean ± standard error; n = 3) of the Triton X-100 soluble Cx43 was assembled into hexameric complexes and 4.4 ± 2.0% (n = 3) of the total Triton

X-100 soluble Cx43 was assembled into intermediate fractions. Treatment of control cells with BFA for 30 min reduced the level of Cx43 assembly to $2.7 \pm 3.8\%$ (mean \pm range; n = 2) of the Triton X-100 soluble pool, with no detectable sub-hexameric intermediates, consistent with previous results for other cell types (Musil and Goodenough 1993). In contrast, $43\beta^2$ cells had more Cx43 assembled into sub-hexameric intermediates $(11.9 \pm 3.8\% (n = 2))$ as compared to hexamers $(6.7 \pm 0.1\% (n = 2))$. Thus, $Cx43/\beta$ -gal inhibited Cx43 hemichannel assembly by \sim 55% as compared to control cells. Given that the intracellular pool of Cx43 was largely in a perinuclear compartment prior to the TGN, this result is consistent with the TGN as the site for hemichannel assembly. The level of inhibition was not complete and was most likely due to leakage of Cx43 to secretory compartments past the Cx43/ β -gal block (Das Sarma et al. 2001). However, these results do not rule out the possibility that some Cx43 hemichannel assembly may have occurred prior to the TGN.

The sub-hexameric, heteromeric complex in $43\beta^2$ cells of intermediate molecular weight was most likely due to formation of a heterodimer between Cx43 and Cx43/ β -gal, since these two connexins were co-immunoisolatable (Das Sarma et al. 2001) and the molecular weight of the intermediate complex (~43 kD + ~159 kD = ~202 kD) was between that of monomeric Cx43 (~43 kD) and hexameric Cx43 hemichannels (~258 kD). However, the resolution for our sucrose gradients was not sufficient to rule out a potential heteromeric complex between Cx43, Cx43/ β -gal and other, as yet unidentified, proteins.

It is tempting to speculate that $Cx43 - Cx43/\beta$ -gal complexes accumulating in the pre-TGN compartment of $43\beta2$ cells reflect a bona fide intermediate in the Cx43 assembly pathway. However, since the mechanism for $Cx43/\beta$ -gal retention in a pre-TGN compartment remains undefined, we cannot be certain that $Cx43 - Cx43/\beta$ -gal complexes reflect an assembly intermediate involved in oligomerizing Cx43 alone. A likely alternative is that the intracellular pool of $Cx43 - Cx43/\beta$ -gal complexes may be an

intermediate in transit to a degradation quality control pathway (VanSlyke et al. 2000), since we cannot rule out the possibility that these pre-TGN connexin complexes were induced by $Cx43/\beta$ -gal. Despite these shortcomings, the finding that $Cx43/\beta$ -gal inhibited Cx43 transport in the Golgi apparatus make it a useful tool, since this is an extremely difficult part of the secretory pathway to examine pharmacologically or through molecular approaches. Given that there are seven distinct Golgi subcompartments, including the ER-Golgi intermediate compartment (ERGIC) and TGN (Marsh et al. 2001), it seems plausible that Cx43 assembly into hemichannels may involve intermediates formed in different intracellular compartments. This is also suggested by transient Cx43 phosphorylation events that occur at the level of the Golgi apparatus (Laird et al. 1995). Nonetheless, putative stable sub-hexameric Cx43 complexes would likely be highly transient and unstable intermediates in connexin oligomerization, since native sub-hexameric connexin intermediates have not been successfully isolated to date. Also, it seems plausible that putative Cx43 assembly intermediates may require co-factors that are not stable under the solubilization conditions currently used to analyze connexin assembly. Further analysis of Cx43 assembly will benefit from the use of constructs containing specific retention motifs for targeting to specific intracellular compartments, such as the ER or

ERGIC, which enable the trapping of assembly intermediates in intact cells.

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