In polarized epithelial cells, subcellular sorting or trafficking of many materials, including membrane proteins and secretory proteins, is controlled by complex mechanisms as yet unknown.\(^1\) It is believed that the direction taken by the secretory protein, either apical or basolateral, is decided by the trans-Golgi network (TGN) and, following this, transport to the appropriate site is carried out by vesicle trafficking.\(^2,3\) Sorting events are regulated by mutual interaction of sorting signal(s) on protein and cellular component(s) involved in the process.\(^4\)

Some sorting signals have been characterized. For instance, the basolateral sorting of integral membrane proteins is regulated by interaction of the tyrosine-based signal sequence of their cytoplasmic domain with \(\mu 1 B,5,6\) a subunit of adaptor protein (AP) complexes which assemble the clathrin coat and play a role in the trafficking of the clathrin-coated vesicles.\(^9\) The basolateral sorting of transferrin receptors (TIR)\(^10\) and low-density lipoprotein receptors (LDLR)\(^9\) is unregulated in LLC-PK\(1\) cells,\(^10,11\) being deficient in \(\mu 1 B,5,6\) and is respected in LLC-PK\(1\) cells expressing exogenous \(\mu 1 B,10\).

Recently, we have reported that the secretion polarity of interferon-\(\beta\) (IFN-\(\beta\)), a typical secretory protein, in the mouse epithelial squamous cell carcinoma, Pam-T, and the canine renal epithelial cell line, MDCK (type I), is different from the previous results of Pam-T\(^12\) and MDCK (type I) cells.\(^13\)

In this report, we investigated the secretion polarity of human IFN-\(\beta\) (HuIFN-\(\beta\)) and the subcellular localization of EGFP-tagged HuIFN-\(\beta\) (HuIFN-\(\beta\)-EGFP) in another type of epithelial cell line, LLC-PK\(_1\), comparing with the previous results of Pam-T\(^12\) and MDCK (type I) cells.\(^13\)

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**MATERIALS AND METHODS**

**Cell Lines, Plasmids, Transfection and Establishment of Stable Lines** LLC-PK\(_1\) cells (ATCC CL101), a porcine renal proximal tubule cell line, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (GIBCO-Invitrogen Co., Carlsbad, CA, U.S.A.), LLC-PK\(_1\) (\(\mu 1 B\) cells)\(^9\) harboring the human \(\mu 1 B\) gene were donated by Hiroshi Ohno (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan). Mouse fibroblast L and human amnion-derived FL cells, used for IFN biossay, have been described previously.\(^14\)

The expression plasmids for HuIFN-\(\beta\) and HuIFN-\(\beta\)-EGFP, dubbed pCMV-HuIFN-\(\beta\) and pCMV-HuIFN-\(\beta\)-EGFP, respectively, have been previously described.\(^12,13,16\) LLC-PK\(_1\) cells, seeded at \(5 \times 10^4\) cells/cm\(^2\) in 6 well plates and cultured for 24 h, were transfected with pCMV-HuIFN-\(\beta\) or pCMV-HuIFN-\(\beta\)-EGFP (3 \(\mu g/ml\) complexed with a cationic liposome, Lipofectamine 2000 (GIBCO-Invitrogen) (10 \(\mu g/ml\)). Stable transformants were picked up as single cell colonies in the presence of 1 mg/ml Genetin (G418) (Sigma-Aldrich, St Louis, MO, U.S.A.). IFN-\(\beta\) production was examined by biossay (see below) or fluorescence of the EGFP portion by confocal laser scanning microscopy (CLSM) (see below). Relatively strong positive sublines, respectively named LLC-PK\(_1\) (HuIFN-\(\beta\)) and LLC-PK\(_1\) (HuIFN-\(\beta\)-EGFP), were used for further experiments.

**Secretion Polarity Experiments** Cells were seeded on Transwell filters (1-cm\(^2\) culture area) (Costar, Cambridge, MA, U.S.A.) at a density of \(1 \times 10^5\) cells per well and cultured for 4 d to establish impermeable cell sheets. In the case of transient gene expression, cells were treated with the plasmid DNA complexed with Lipofectamine 2000, at the same time.
DNA/liposome doses as described above, from the upper or lower compartment for 4 h at 37°C. Then, the medium in both compartments was changed to growth medium. In the case of stable expression, stable transformants were seeded on Transwell filters and the IFN secretion was examined in a similar way. In either case, a small amount of mouse IFN-β (final concentration about 300 U/ml) was added to the upper compartments as a permeability marker 1 h after the last medium change. Then, 48 h after transfection, the culture fluids in both compartments were separately harvested to measure their IFN activities by bioassay.

**IFN Bioassay** The human and mouse IFN-β activities in the cell culture supernatants were measured in FL and L cells, respectively, and expressed in antiviral units (U) as described previously.12,15)

**Laser Scanning Confocal Microscopy** Cells were seeded on Transwell filters at a density of 1×10^5 cells per well and incubated for 4 d. In the transient gene expression experiments, cells were treated with pCMV-HuIFNβ-EGFP complexed with Lipofectamine 2000, and incubated following a medium change, as described above. Then, 8 h after transfection, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature. Following this, the cells were washed with PBS and permeabilized with 0.1% Triton-X100-PBS for 20 min, followed by treatment with 15 μg/ml RNase A (Roche Diagnostics, Indianapolis, IN, U.S.A.) at 37°C for 30 min. After washing with PBS, the cell nuclei were stained with 0.5 μg/ml propidium iodide (PI) in 0.1 m Tris–HCl (pH 7.4) containing 0.1 m NaCl for 20 min at room temperature. In the stable gene expression experiments, the cells were fixed and the nuclei were stained with PI as described above. The filters were cut out of the holders, mounted on coverslips, and then immersed in 50% glycerol–2.5% DABCO (1,4-diazabicyclo-[2,2,2]octane) (Sigma-Aldrich, St. Louis, MO, U.S.A.)–PBS. The emissions from the optical x–y sections were collected using a confocal microscope (MRC-1024; BioRad, Hercules, CA, U.S.A.) as previously described.13)

The collected x–y image segments were processed to obtain x–z images.

**RESULTS**

At first, secretion profile of HuIFN-β expressed in LLC-PK₁ cells was investigated for stable and transient expression. Figure 1A shows that stably expressed HuIFN-β in LLC-PK₁ cells was secreted to both the apical and basal compartments. These secretion profiles are similar to those seen in Pam-T12) and MDCK (type I) cells.13) When transiently expressed by lipofection from the apical side, HuIFN-β was secreted to both compartments (Fig. 1B), but when transiently expressed by lipofection from the basal side, HuIFN-β was preferentially secreted to the basal side. Thus, the secretion mode of stable HuIFN-β in LLC-PK₁ is similar to that in Pam-T and MDCK (type I), while the transient expression LLC-PK₁ in apical side transfection was different from that in Pam-T and MDCK (type I) cells.12,13)

Since μ1B is considered as a candidate for the responsible molecule(s) of the particular secretion mode of HuIFN-β in LLC-PK₁ cells, we examine the secretion of HuIFN-β in μ1B-expressing LLC-PK₁ (LLC-PK₁(μ1B)) cells.9) As shown in Fig. 2, the secretion profile of HuIFN-β transiently expressed in LLC-PK₁(μ1B) was similar to that in LLC-PK₁ cells, implying that the involvement of μ1B in the secretion mode of HuIFN-β in those cells is negligible.

DISCUSSION

In this report, we investigated the secretion polarity of HuIFN-β in LLC-PK₁ cell layers. Although the constitutive secretion profile is non-polarized similar to that seen in Pam-T12) and MDCK (type I) cells,13) no apical-side transfection-dependent secretion was observed in the LLC-PK₁, cells (Fig. 1B). These results suggest that the secretion mode of tran-
proteins,9,17) we carried out an investigation to see if the expression of HuIFN-

b may be different. Expressed in LLC-PK1 Cell Layers

It is unlikely that the secretion mode of transiently expressed IFN-β is the artifact of transfection with lipofection, because the secretion mode was not affected by concomitant treatment with liposomes alone or empty vector complexed with liposomes from the other side of the IFN-β expression transfection (data not shown). Since μ1B-expressing LLC-PK1 exhibits proper distribution of some membrane proteins,1,7 we carried out an investigation to see if the μ1B expression is related to the secretion of HuIFN-β. However, the secretion polarity of HuIFN-β in μ1B-expressing LLC-PK1 (μ1B) cells was essentially the same as that in LLC-PK1 cells (Fig. 2). Consequently, μ1B expression does not seem to be involved in HuIFN-β secretion polarity.

Confocal imaging analysis of the subcellular localization of HuIFN-β-EGFP revealed that there is apparently no significant difference in stable and transient expression following apical or basal transfection in LLC-PK1 cells (Fig. 3C); in the case of both forms of expression, HuIFN-β-EGFP was distributed around the cell nuclei, probably at Golgi/TGN as suggested in the previous report.13 In LLC-PK1 cells, stably expressed HuIFN-β and transiently expressed HuIFN-β following apical side transfection are transported from Golgi/TGN to the extracellular compartment via the same pathway, while transiently expressed HuIFN-β following basal side transfection are transported from Golgi/TGN to the basal compartment via a different secretory pathway. Furthermore, transcription fields of exogenous genes in the nucleus might be different between transient and stable expression, which might doom the secretion in a cell type-dependent manner by yet unknown mechanisms. In addition, certain cellular molecule(s) other than μ1B that is expressed in Pam-T and MDCK (type I) but not in LLC-PK1, or expressed inversely might involve with the secretion route of HuIFN-β.

Taken together, these results suggest that the secretion polarity of the same secretory protein, as far as IFN-β is concerned, in polarized epithelial cells is different in the case of stable and transient expression and that this phenomenon is regulated by sorting events at post-Golgi/TGN in a cell type specific manner, although the precise mechanisms are currently unknown.

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