Determining subcellular localization of novel drug targets by transient transfection in COS cells

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Abstract

Genomics-based approaches are increasingly being used to identify disease-associated genes that represent potential new drug targets. As a first step in the validation of genes of unknown function, we describe a method for rapidly determining the subcellular localization of the gene product. If an immunotherapeutic approach is being considered, it is of particular interest to identify targets that are either on the cell-surface or secreted. Transient expression in COS cells combined with immunofluorescent staining provides a semi-high throughput method for determining the subcellular localization of multiple targets in parallel. COS cells are ideal for this purpose since: (i) they transfect easily; (ii) the high levels of expression that can be achieved transiently allow detection after 24 h; and (iii) the relatively large size and spread morphology of these cells allows the subcellular organelles to be easily visualized. To evaluate the system, we show prototype staining patterns for known cytoplasmic, secreted, Golgi-associated, endoplasmic reticulum-associated, and plasma membrane proteins, as well as data for novel targets. The localization of novel secretory and cell-surface proteins as determined by immunofluorescent staining, was confirmed by independent methods.

Introduction

The use of DNA microarrays and other new technologies has enabled the identification of potential drug targets based on their expression profiles. Genes that are upregulated or overexpressed in disease tissues compared to healthy tissue are rapidly being identified. Most of these disease-associated genes are uncharacterized, and require further functional validation to establish their usefulness as either diagnostic makers or targets for therapeutic intervention. Determining the subcellular localization of the gene product is an important first step in this process. In particular, because of recent interest in therapeutic antibodies, it is important to determine if a potential target is a cell-surface or secreted molecule that would be accessible to an immunotherapy approach. Once a full-length sequence encoding the complete open reading frame (ORF) has been assembled, it can be examined for the presence of a signal sequence in the case of secreted proteins, or of hydrophobic transmembrane domains in the case of membrane proteins. However, prediction programs are not always accurate, and the presence of a hydrophobic domain does not ensure localization on the plasma membrane. Subcellular localization must subsequently be confirmed experimentally.

Cell-surface and secreted proteins enter and traverse the secretory pathway in order to reach their final destinations. The biosynthetic pathway begins with co-translational translocation of nascent chains across the membrane of the endoplasmic reticulum (ER), followed by protein folding, processing, and subunit assembly. The protein then exits the ER and is transported by vesicular transport through the Golgi Apparatus to the cell surface (for review see Rothman, 1994; Rothman and Wieland, 1996). Immunofluorescent detection of an expressed protein in transit through these compartments can be used as a means of identifying cell-surface or secreted proteins.
Transient transfection in COS cells combined with immunofluorescent staining provides a method for rapidly determining the subcellular localization of novel drug targets. COS cells were selected for this purpose because their size, morphology, and tight adherence to plastic or glass surfaces allow the subcellular organelles to be easily stained and visualized. In addition, constitutive expression of SV40 T-antigen increases the copy number of plasmids containing an SV40 origin of replication (Mellon et al., 1981), allowing extremely high levels of expression to be achieved transiently. Expressed proteins can therefore be detected easily in 24 h.

**Methods**

**Expression plasmids and transfections**

Cloned cDNAs were inserted into a modified version of the mammalian expression vector, pEF6/V5-His from Invitrogen (Carlsbad, CA, USA) in which the V5-His epitope tag sequences were replaced with the FLAG epitope, DYKDDDDK. To enable high-throughput cloning, the FLAG sequence was placed downstream of the cloning site such that the insertion of a PCR-amplified cDNA creates an in-frame fusion of the ORF with the FLAG sequence. Plasmid DNA for transfection was prepared using a kit from Qiagen (Valencia, CA, USA). COS-7 cells (Gluzman, 1981) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Serum-free medium (IS293) was from Irvine Scientific (Santa Ana, CA 92705). Transfections were carried out using Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, for each 6-well, 4.5 µl of Fugene-6 and 0.75 µg DNA were diluted into 100 µl DMEM and added to the cells.

**Antibodies**

The anti-FLAG antibody was from Sigma, St. Louis, MO, USA. Rabbit anti-Grp78 was from Stressgen Biotechnologies Corp (Victoria, BC Canada). Anti Syntaxin 6 and anti-GM130 were from Transduction Laboratories (Lexington KY, USA). ER tracker and ALEXA-conjugated anti-mouse or anti-rabbit IgG was from Molecular Probes. The anti-CD55 antibody was from Caltag (CA, USA) and anti-human IgG Fc was from Jackson Immunoresearch (PA, USA).

**Immunofluorescent staining**

For immunofluorescent staining experiments, cells were grown and transfected on glass coverslips in 6-well or 12-well dishes. Approximately 24 h following transfection, the cells were fixed for 4 min with 4% formaldehyde in phosphate-buffered saline (PBS) and then permeabilized for 4 min with 0.5% Triton X-100 in PBS. The cells were then covered with blocking solution (PBS containing 5% fetal bovine serum and 2% bovine serum albumin) and incubated for 1h at room temperature. The blocking step was followed by incubation for 1–2 h at room temperature with mouse anti-FLAG IgG (0.6 µg ml⁻¹ in blocking solution) or with other antibodies as indicated. The cells were then washed twice with PBS, incubated for 30–60 min with ALEXA-conjugated anti-mouse IgG (1.3 µg ml⁻¹), washed three times with PBS and mounted on glass slides using Permafluor aqueous mounting medium from Immunon (Pittsburgh, PA, USA).

**Cell surface biotinylation and Western Blots**

Cells were grown and transfected in 6 cm or 10 cm dishes. The medium was replaced with serum-free medium 18 hours after transfection. For every experiment, a non-transfected control dish was carried through the same protocol. Cells were harvested 48 hours after transfection using Cell Dissociation buffer (Gibco BRL) and washed three times with PBS to remove any contaminating proteins and cell debris. Cells (~5 × 10⁶) were suspended in 0.5 ml PBS and 100 µl of 1 mg ml⁻¹ Sulfo-NHS-SS-Biotin (Pierce) was added. After 30 min, the cells were washed once with TBS (25 mM Tris-HCL pH 7.5, 150 mM NaCl) to stop the reaction, and then gently washed three times with PBS. The cells were then lysed with 1% Triton X-100 in TBS containing a protease inhibitor cocktail (CalBiochem, San Diego, CA). After a 15 minutes incubation, the cell lysates were centrifuged at 15,000 × g for 10 minutes and the resulting supernatant was incubated with 50 µl of streptavidin-conjugated beads (Pierce, Rockford, IL) for 30 minutes at room temperature. The beads were recovered by centrifugation, washed once with RIPA buffer (150 mM NaCl, 50 mM Tris-HCL pH 7.8, 1% sodium desoxycholate, 0.1% SDS, 25 mM imidazole, 1.5% Triton X-100), once with 25 mM Tris-HCL, pH 7.5, 500 mM NaCl and once with TBS. The biotinylated, streptavidin-bound proteins were eluted by boiling the beads with 50 µl Tris-glycine sample buffer (NoveX, Invitrogen, Carlsbad, CA) for 5 minutes. The eluted proteins were