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

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Received 10 January 2001; accepted 2 July 2001

Key words: immunofluorescent staining, surface biotinylation, transient transfection

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 μ l of Fugene-6 and 0.75 μ g DNA were diluted into 100 μ l 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 1 h at room temperature. The blocking step was followed by incubation for 1–2 h at room temperature with mouse anti-FLAG IgG (0.6 μ g 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 μ g 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 ($\sim 5 \times 10^6$) were suspended in 0.5 ml PBS and 100 μ l 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 $\times$ g for 10 minutes and the resulting supernatant was incubated with 50 μ l 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 pH7.8, 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 μ l Tris-glycine sample buffer (Novex, Invitrogen, Carlsbad, CA) for 5 minutes. The eluted proteins were

Figure 1. Secretory pathway organelles in COS cells. Cos cells, grown on glass cover slips, were fixed and permeabilized as described in Methods and then stained with (a), anti-GRP78 to visualize the ER; (c) anti-GM130 to visualize the cis-Golgi; or (e), anti-syntaxin 6 to visualize the TGN, followed by Alexa-conjugated anti-rabbit or anti-mouse secondary antibodies; (d, f), the immunofluorescent staining patterns in (c) and (e) were superimposed on the corresponding phase contrast images to show the juxtanuclear position of the Golgi complex; (b), the ER, visualized using a fluorescent dye, ER-tracker (Molecular Probes), selective for the ER.

separated on a 8–16% SDS-PAGE gel (Novex) and electrotransferred onto PVDF membrane (BioRad). After blocking with milk, blots were incubated for one hour with hydrogen peroxidase (HRP)-conjugated anti-Flag-IgG (Sigma) and visualized by an enhanced chemiluminescence (ECL) detection system (Amersham Corp., Piscataway, NY) as described by the manufacturer.

Immunoprecipitation

Cells were grown and transfected as described above. Non-transfected cells served as negative controls. The cells and medium were harvested 24 or 48 hours after transfection. The cells were washed with PBS and lysed with 1% Triton X-100 in TBS as described above. The cell lysate and culture medium were incubated overnight with anti-Flag-IgG conjugated to Agarose beads (Sigma) that had been pretreated and equilibrated as described by the manufacturer. After incubation, the beads were washed twice with TBS and the bound proteins were eluted by two sequential incubations with 100 μ l Flag peptide (0.1 mg ml⁻¹) for ten minutes. The elutions were combined, concentrated in a SpeedVac to 30 μ l and loaded

onto an appropriate SDS-PAGE gel. The separated proteins were electrotransferred onto a PVDF membrane, incubated for one hour with HRP-conjugated anti-Flag-IgG (Sigma) and visualized by enhanced chemiluminescence (ECL) detection.

Results

Morphology of secretory pathway organelles in COS-7 cells

In order to be able to identify secretory and cell surface proteins in COS cells, we first visualized the compartments of the secretory pathway by staining cells with specific organelle markers (Figure 1). The ER was visualized either with an antibody to GRP78 (BIP), a resident protein of the ER, or with the fluorescent dye, ER-tracker. The ER surrounds the nucleus and has a feathery or lacy appearance (Figures 1a, b). The cis-Golgi was visualized with anti-GM130 (Nakamura et al., 1997) and appears as a compact cluster of tubules localized asymmetrically on one side of the nucleus (Figures 1c, d). The Trans-Golgi Network (TGN) was visualized using an antibody to syntaxin

Figure 2. Immunofluorescent staining patterns of transiently expressed proteins known to be targeted to different subcellular compartments. COS-7 cells were transfected with expression vectors encoding: (a), a truncated, secreted CD55 extracellular domain (Caras et al., 1989) fused to an IgG Fc domain; (b), full-length CD55, a GPI-anchored cell-surface protein (Davitz et al., 1986); or (c), an IgG Fc domain that, lacking a signal sequence, is expressed as a cytoplasmic protein (unpublished observations). The expressed proteins were detected either with an anti-CD55 antibody or with an anti-Fc antibody.

Figure 3. Immunofluorescent staining patterns of targets predicted to be secreted proteins: (a) Target 1; (b) Target 2; (c) Target 3; (d) Target 4.

6 (Bock et al., 1996) and appears to partially overlap the cis-Golgi, but in addition contains diffuse structures that radiate outwards towards the cell periphery (Figures 1e, f).

Prototype staining patterns for different expressed proteins in COS-7 cells

We next tested whether transiently expressed proteins whose subcellular localization had previously been characterized, and which were known to be either secreted, on the cell surface, or localized in the cytoplasm, could be recognized and differentiated based on their immunofluorescent staining patterns in COS cells (Figure 2). As expected, staining of the secretory protein is limited to the ER and Golgi (Figure 2a). In contrast, the pattern for the cell-surface protein (Figure 2b) is characterized by staining of the plasma membrane surrounding each cell in addition to the ER and Golgi, the latter revealing protein en route to the cell surface. The cytoplasmic pattern (Figure 2c) is characterized by diffuse staining evenly distributed throughout the cytoplasm.

Subcellular localization of putative secretory proteins

To enable the protein products of novel or uncharacterized cDNAs to be detected, the ORFs were amplified by PCR and cloned into an expression vector that by design, adds a sequence encoding the 8-amino acid FLAG epitope (DYKDDDDK) to the COOH-terminus of the predicted protein. The recombinant proteins can then be detected by immunofluorescent staining with an anti-FLAG antibody. Figure 3 shows the staining patterns we obtained for four targets predicted to be secreted proteins, each of which showed a different, distinctive pattern. Targets 1 and 2 have patterns consistent with a secreted protein, where the expressed protein is detected primarily in the ER and Golgi (Figures 3a and b). Target 3 is visible in the Golgi, indicating that this protein has entered the secretory pathway, as well as in numerous small vesicles, possibly transport vesicles, scattered throughout the cell (Figure 3c). Target 4, which was predicted to contain a non-cleavable amino-terminal signal sequence, appears to be localized primarily in the cell nucleus, with some protein in the cytoplasm (Figure 3d). This result suggested that Target 4, despite the sequence prediction, is not a secretory protein.

To verify the above conclusions, we immunoprecipitated the recombinant proteins from both the cell lysates and culture media of transfected COS cells

and performed a Western blot analysis (Figure 4). Molecular species corresponding to Targets 1, 2 and 3 (Figures 4a–c) were detected in both the culture media and cell lysates of transfected cells, confirming that these are indeed secreted proteins. In all three cases, the secreted species, which presumably corresponds to a fully glycosylated form, was larger than the cell-associated species. In agreement with the immunofluorescent staining pattern, Target 4 (Figure 4d) was detected only in the cell lysate, confirming that it is not a secretory protein. Interestingly, the staining patterns observed for each of the three secreted targets were not identical (Figure 3), suggesting that secretory proteins do not move together through the cell by a bulk flow process, but rather, each protein appears to be handled differently by the cell machinery (Klumperman, 2000).

Subcellular localization of putative cell-surface proteins

Figure 5 shows the staining patterns obtained for several targets predicted to be transmembrane proteins with possible localization on the plasma membrane. Targets 5 and 6 are predicted to be type I membrane proteins, having a cleavable amino-terminal signal sequence and a single TM. Target 7 is a type III membrane protein with 6 TMs, and Targets 8 and 9 are members of the G-protein-coupled receptor (GPCR) family, which have 7 TMs. The staining patterns in COS cells indicate that Targets 5, 6, 7, and 8 are expressed on the cell surface (Figures 5a–d). Targets 5 and 8 (a and d) appear to be uniformly distributed on the plasma membrane while Target 7 (c) is prominently expressed on microspikes and filopodia that protrude from the plasma membrane. Target 6 (b) has an unusual localization in that it appears to be highly concentrated on pseudopodia-like projections of the cell. One of the two GPCRs, Target 9 (e), is detected only in the ER and not on the plasma membrane, suggesting that it enters the secretory pathway but is then retained in the ER and does not traffic to the cell surface. This target is a putative olfactory receptor and may require specific chaperones for insertion into the plasma membrane (Gimelbrant et al., 1999).

To verify the above results and confirm the cell surface localization, transfected cells were labeled with a non-permeant, biotinylation reagent (Kahne and Anson, 1994; Cole et al., 1984). Only those protein domains that are exposed on the cell surface are accessible to this reagent and can be labeled by it. The

Figure 4. Western blot analysis of cell lysates and culture supernatants from cells transfected with: (a), Target 1; (b), Target 2; (c), Target 3 and (d), Target 4. For each experiment, cell lysates are in Lane 1 and culture supernatants from either non-transfected or transfected cells in lanes 2 and 3 respectively.

biotinylated proteins were purified on streptavidin-coated beads and analyzed on a Western blot with an anti-FLAG antibody (Figure 6). Specific detection of the target proteins by this method verifies that the target is exposed on the cell surface and confirms the plasma membrane localization as assessed by immunofluorescent staining.

Discussion

The above procedure for determining the subcellular localization of novel drug targets is simple and inexpensive and yields reliable results in 1 to 2 days. Multiple targets can be analyzed in parallel in a semi-high throughput fashion, the limiting factor being the cloning of full-length ORFs. The 8-amino acid epitope tag that is added to the COOH-terminus of the expressed protein to enable its detection, is less likely to perturb the normal trafficking and localization of the target protein than alternative strategies involving fusion proteins, for example using green fluorescent protein (Simpson et al., 2000).

One potential concern is that overexpression of a protein relative to normal physiological levels, could alter the subcellular localization and lead to erroneous conclusions. However, we have expressed approximately 20 different known proteins using this system and have so far not found this to be the case. For example, when we overexpressed furin, a resident protein of the TGN, all of the expressed protein remained cell-associated and correctly localized in the TGN. We were unable to detect any release of furin into the culture medium (data not shown). Similarly, overexpression and detection of several known cell surface, GPI-linked or secreted proteins, has so far not revealed aberrant trafficking or localization as a result either of the overexpression, or of the presence of the COOH-

terminal epitope tag (data not shown). In comparing different cell surface or secreted proteins, we have noted differences in the relative staining intensities of the different sub-compartments of the secretory pathway. For example, in some cases a relatively large fraction of the overexpressed protein accumulated in the ER, possibly due to inefficient folding, which hinders exit from the ER. However, this did not affect our ability to correctly identify secreted or cell-surface proteins, based on their presence at the final destination. In addition, we have found that localization results obtained in COS cells are consistent with results obtained using diverse cell types, including mouse fibroblasts, Chinese hamster ovary cells, and various human cancer lines (data not shown).

The methods described in this report allow an investigator to assign a localization that is cell-surface, secreted, cytoplasmic or nuclear, with a high degree of accuracy. However, if a putative transmembrane or secreted target is observed to be localized entirely in the ER, a number of interpretations are possible. One possibility is that the protein is a resident ER protein. Such proteins generally contain a known ER-retention signal such as the KDEL motif, although novel retention signals may exist. An alternate possibility is that the protein has misfolded or misassembled, and is then prevented from exiting the ER by a quality control mechanism (reviewed by Brodsky and McCracken, 2000). When ER retention is encountered, as in the case of Target 9 above, alternative methods must be used to assign an accurate localization. Replacing COS cells with a cell line that endogenously expresses the gene in question might solve the problem by supplying either the missing components of a multimeric complex, or the missing chaperones required for correct folding. Despite this caveat, transient transfection in COS cells as described, has proved to be a reliable

Figure 5. Immunofluorescent staining patterns of targets predicted to be membrane proteins: (a) Target 5; (b) Target 6; (c) Target 7; (d) Target 8; (e) Target 9. Targets 5–8 show cell-surface staining while Target 9 is retained in the ER. Target 7 (c) is expressed on microspikes and fillopodia (indicated by the arrow); Target 6 (b) is highly concentrated on pseudopodia-like projections of the cell (arrow).

Figure 6. Confirmation of cell-surface localization by surface biotinylation and Western analysis. Cells expressing (a), Targets 5; (b), Target 6; or (c), Target 8, were biotinylated and analyzed as described in Methods. Non-transfected control samples are in lane 1 and transfectants in lane 2.

method for determining the subcellular localization of most novel drug targets.

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