Current methods to detect protein-protein interactions are either laborious to implement or not adaptable for mammalian systems or in vitro methods. By adding a peroxisomal targeting signal (PTS) onto one protein, binding partners lacking a targeting signal were co-transported into the peroxisomes in a "piggy-back" fashion, as visualized by confocal and electron microscopy. A fragment of colicin E2 and its tightly interacting immunity protein, ImnE2, were both expressed in the cytosol. When either one contained a PTS tag, both proteins were co-localized in the peroxisomes. The cytokine-independent survival kinase (CISK) containing a PTS tag was not efficiently targeted to the peroxisomes unless the Phox homology (PX) domain, attaching the protein to endosomal membranes, was removed. However, PTS-tagged CISK with deleted PX domain was able to direct 3-phosphoinositide-dependent protein kinase-1 (PDK-1) into the peroxisomes. This demonstrates that the two proteins interact in vivo. Mutating Ser486, which is phosphorylated in activated CISK, to Ala prevented the interaction, indicating that CISK and PDK-1 interact in a phosphorylation-dependent manner. The method therefore allows assessment of protein-protein interactions that depend on post-translational modifications that are cell-specific or dependent on the physiological state of the cell.
all components do not need to possess a PTS but can be imported in a "piggy-back" fashion. This demonstrates the remarkable import capacity of these organelles (16, 17).

We exploited these peroxisomal features and targeted one protein to the peroxisomes by adding a PTS. Co-import of an interacting partner without a PTS was observed by confocal microscopy. Demonstration of such peroxisomal co-localization through evidence that the protein binds to each other in the cytosol of the living cell before the protein complex traverse the peroxisomal membrane. After establishing that this principle was feasible, by employing two proteins that interact with very high affinity, we provide evidence for the binding between CISK and PDK1. Additionally, we show that this binding is dependent on the phosphorylation of the regulatory domain of CISK. The ability to assay for interactions dependent on post-translational modifications is a great advantage of the present assay system, in addition to its simplicity and affordability.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-Myc antibodies were from the 9E10 hybridoma (18). Human anti-EEA1 serum was a gift from Ban-Hock Toh. Rabbit anti-catalase was purchased from Calbiochem. Anti-mouse lissamine-rhodamine, anti-rabbit Cy5, and anti-human rhodamine antibodies were purchased from Jackson ImmunoResearch.

Cells and Transfections—HeLa and/or COS-1 cells were propagated in Dulbecco’s modified essential medium with 10% fetal calf serum in PBS. The cells were then incubated with Subsequently, the cells were washed in PBS and blocked for 20 min and the peroxisomal membrane was then permeabilized by incubation in the cytosol of the living cell before the protein complex traverse the peroxisomal membrane. After establishing that this principle was feasible, by employing two proteins that interact with very high affinity, we provide evidence for the binding between CISK and PDK1. Additionally, we show that this binding is dependent on the phosphorylation of the regulatory domain of CISK. The ability to assay for interactions dependent on post-translational modifications is a great advantage of the present assay system, in addition to its simplicity and affordability.

In Vitro Phosphorylation—Recombinant MBP and MBP fusion proteins of CISK were expressed and purified in E. coli and dialyzed into kinase assay buffer (20 mM Hepes, pH 7.4, 10 mM MgCl2, 10 mM MnCl2, and 0.1% 2-mercaptoethanol). In the phosphorylation assay the recombinant proteins were added to a kinase buffer containing ATP (Sigma), [γ-32P]ATP (Amersham Biosciences) and thereafter incubated with PDK-1 active (Upstate Biotechnology), as suggested by the manufacturers. After 30 min at 30 °C, SDS-stop solution was added to the samples and run on SDS-PAGE. After fixation and drying, the gel was scanned by a PhosphorImager (Amersham Biosciences).

Electron Microscopy—Cells were fixed in a mixture of 2% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. Cell preparation for cryoimmunocytochemistry was performed according to Ref. 21, and double labeling was performed as described in Ref. 22.

RESULTS

Demonstration of Protein-Protein Interaction by Co-transport to Peroxisomes—To evaluate the ability of peroxisomal targeting to assess protein-protein interactions, we targeted a fragment of colicin E2, which contains the ImmE2 binding domain (BD), to the peroxisomes by adding a PTS-1 tag (Fig. 1A, upper half) and tested if peroxisomal co-import occurred in cells co-transfected with both interacting proteins (Fig. 1A, lower half). Myc-tagged ColBD containing a C-terminal PTS-1 (Myc-ColBDpts) was constructed and transiently transfected into HeLa cells. Co-localization between the peroxisomal marker catalase and Myc-ColBDpts was determined by merging the confocal images representing catalase (blue) and Myc-ColBDpts (red) (Fig. 1B). Peroxisomes containing Myc-ColBDpts were then observed as purple-colored dots in the merged image. Virtually all the organelles that stained for the peroxisomal marker catalase were also positive for Myc-ColBDpts demonstrating that Myc-ColBDpts was efficiently recruited to the peroxisomes.

Next HeLa cells were co-transfected with Myc-ColBDpts and ImmE2 containing a GFP tag (GFP-ImmE2). After merging the relevant confocal images we could observe extensive co-localization of the proteins, here observed as a yellow punctuate pattern (Fig. 1B). To test that the yellow staining after merging red and green images represent specific co-localization and not accidental overlap of green- and red-stained structures, we have introduced a technique where the images are merged askew by a distance of approximately one peroxisome diameter in the relevant image. If the yellow-stained peroxisomes then reverts to red and green this ensures specific co-localization, as seen for the images representing Myc-ColBDpts and GFP-ImmE2 that were merged 9 pixels askew (Fig. 1B). Here yellow staining was completely reversed to red and green providing evidence that colicin E2 interacts with its immunity protein, ImmE2, in the living cell and that the peroxisomal targeting mechanism is feasible (Fig. 1B). When there is a higher level of background staining, such askew merging is extremely useful to yield a clear “yes or no” answer, as seen in the example described below.

The PTS-1 tag could also be added to the binding partner, in this case the immunity protein. We constructed GFP-ImmE2pts and repeated the co-localization experiment described above with Myc-ColBD lacking a PTS signal. GFP-ImmE2pts was targeted to the peroxisomes with equal efficiency as Myc-ColBDpts and additionally recruited Myc-ColBD to virtually all the peroxisomes stained for catalase in co-transfected cells (data not shown). This experiment demonstrates that this alternative rice verso approach is indeed feasible, which increases the versatility of the assay.
To ensure that these proteins are not normally localized to the peroxisomes, we transfected both HeLa and COS-1 cells with constructs lacking the PTS tag. Neither GFP-ImmE2 nor Myc-ColBD co-localized with the peroxisomal marker catalase (blue), which yields purple-colored peroxisomes upon co-localization. PTS-tagged proteins are able to piggy-back interacting proteins lacking a peroxisomal targeting signal into the peroxisomes. After co-transfecting cells with Myc-Col BDpts and GFP-tagged ImmE2 protein (GFP-ImmE2), evidence for an interaction is provided if merging the confocal images representing Myc-Col BDpts (red) and GFP-ImmE2 (green) gives a yellow peroxisomal staining pattern. B, HeLa cells grown on coverslips were co-transfected with Myc-Col BDpts and GFP-ImmE2 and incubated at 37 °C for 24 h. The cells were depleted of cytosolic material by permeabilization of the plasma membrane by digitonin to reduce background staining prior to fixation in 3% paraformaldehyde. The digitonin resistant peroxisomal membranes were then permeabilized with Triton X-100 to allow the immunostaining antibodies access to the peroxisomal lumen. Rabbit anti-catalase and mouse anti-Myc antibodies followed by anti-rabbit Cy5-conjugated and anti-mouse lissamine-rhodamine antibodies were used for immunostaining of the peroxisomal marker catalase and Myc-ColBDpts, respectively. The samples were analyzed by using a Leica confocal microscope. C, GFP-ImmE2 was transfected into HeLa and the cells treated as described in B except that the immunostaining was performed using a rabbit anti-catalase primary antibody followed by an anti-rabbit-Cy3-labeled secondary antibody.

Complete Translocation into the Peroxisomal Lumen—To ensure that the protein complex had been translocated into the peroxisomal lumen, rather than being attached as aggregates at the organelle surface, we omitted the Triton X-100 treatment during the procedure preparing for immunofluorescence. Triton X-100 is used to permeabilize the peroxisomal membranes after the cells have been depleted of cytosolic material by digitonin treatment and fixed in paraformaldehyde. Omitting Triton X-100 will therefore leave the organelle membrane intact and its lumen inaccessible for immunostaining antibodies. In HeLa cells co-transfected with GFP-ImmE2 and Myc-Col BDpts, only the autofluorescent GFP-ImmE2 showed a typical peroxisomal staining pattern (Fig. 2). The absence of both catalase and Myc-Col BDpts staining indicates that the peroxisomal membrane was intact, since the antibodies could not bind to their respective proteins. These results therefore pro-
vide evidence that the Myc-ColBDpts-GFP-immE2 complex had been completely translocated into the peroxisomal lumen. HeLa cells grown on coverslips were co-transfected with Myc-Col BDpts and GFP-ImmE2 and grown at 37 °C for 24 h. The cells were depleted of cytosolic material by digitonin treatment and fixed in 3% paraformaldehyde. Conventional Triton X-100 treatment was omitted, leaving the peroxisomal membranes intact. Double staining using rabbit anti-catalase and mouse anti-Myc antibodies followed by anti-rabbit Cy5-conjugated and anti-mouse lissamine-rhodamine antibodies was then performed and the samples analyzed in a Leica confocal microscope.

Fig. 2. The Myc-ColBDpts-GFP-immE2 protein complex is completely translocated into the peroxisomal lumen. HeLa cells grown on coverslips were co-transfected with Myc-Col BDpts and GFP-ImmE2 and grown at 37 °C for 24 h. The cells were depleted of cytosolic material by digitonin treatment and fixed in 3% paraformaldehyde. Conventional Triton X-100 treatment was omitted, leaving the peroxisomal membranes intact. Double staining using rabbit anti-catalase and mouse anti-Myc antibodies followed by anti-rabbit Cy5-conjugated and anti-mouse lissamine-rhodamine antibodies was then performed and the samples analyzed in a Leica confocal microscope.

Fig. 3. PTS-1 tagging a truncated version of CISK, lacking the PX domain, gave efficient relocation from the early endosomal membranes to the peroxisomes. A, GFP-CISK was transfected into HeLa cells and incubated at 37 °C for 16 h. Cytosolic material was depleted using 0.05% Saponin and the cells fixed in 3% paraformaldehyde. Immunostaining was performed using a human anti-EEA-1 primary antibody followed by an anti-human-rhodamine secondary antibody. B, HeLa cells were grown on coverslips and transfected with GFP-CISKΔPXpts. After incubation at 37 °C for 24 h the cells were depleted of cytosolic material by digitonin treatment and fixed in 3% paraformaldehyde. The peroxisomal membranes were permeabilized with Triton X-100 to give access to the immunostaining antibodies. Rabbit anti-catalase followed by an anti-rabbit-Cy3-labeled secondary antibody were used for immunostaining, and the images were collected using a Leica confocal microscope. C, HeLa cells were grown on coverslips and transfected with GFP-CISK, GFPCISKpts, or GFP-CISKΔPXpts. Immunostaining was performed as described in B to analyze 150 transfected cells representing each construct for peroxisomal targeting.

Peroxisomal Targeting of CISK pts Is Strongly Inhibited by the Presence of a PX Domain—To determine whether the peroxisomal targeting system could be employed to study protein-protein interaction between CISK and PDK-1, a PTS tag was added to GFP-CISK (GFP-CISKpts). However, we did not ob-
serve the usual efficient peroxisomal recruitment. Only a few of
the transfected cells showed peroxisomes containing GFP-
CISKpts, while in the majority of cells GFP-CISKpts was re-
tained at the endosomal membranes (data not shown).

Peroxisomal targeting may be difficult or impossible if one or
both of the interacting proteins are associated with or inserted
into cellular membranes. GFP-CISK is targeted to the early
endosomal membranes as shown in Fig. 3A and previously
reported by others (1, 6). This localization is mediated by the
attachment of the PX domain to specific phosphoinositides on
the early endosomal membranes (5, 6).

To circumvent this problem we made a construct expressing
a truncated version of CISK lacking the lipid binding PX do-
main (GFP-CISK/H9004PXpts). A dramatic increase in peroxisomal
targeting was observed in HeLa cells transfected with GFP-
CISK/H9004PXpts compared with the GFP-CISKpts transfectants
(Fig. 3B and C). This indicates that the PX-domain retains
CISK at the endosomal membranes and that the truncated
protein is now readily targeted to the peroxisomes.

CISK and PDK1 Interact in Vivo—To assess for a binding
between CISK and PDK-1 we co-transfected both COS-1 (Fig.
4) and HeLa cells (data not shown) with Myc-PDK1 and
GFP-CISKΔPXpts. After immunolabeling we observed that
Myc-PDK1 co-localized with GFP-CISKΔPXpts in the peroxi-
somes. This indicates that GFP-CISKΔPXpts had “piggy-
backed” Myc-PDK1 into the peroxisomes due to an interac-
tion between the proteins in the cytosol. Electron microscopy
demonstrated that PDK-1 lacking a PTS tag was present
inside the peroxisomes in cells co-transfected with GFP-
CISKΔPXpts and Myc-PDK1 (Fig. 5). This observation sup-
ports that CISK-PDK-1 interact, and in addition it indicates
that the protein complex had completely translocated into the
peroxisomal lumen.

CISK and PDK1 Interact in a Phosphorylation-dependent
Manner—Phosphorylation of CISK Ser486 in the hydrophobic
motif has been proposed to be required for PDK1 binding (9,
11). We mutated Ser486 to alanine (CISKΔPXptsS486A) to abol-
ish this phosphorylation step of CISK and used the peroxisomal
targeting assay to determine whether the mutation abolished
the interaction with PDK-1. When co-transfecting GFP-CISKΔ
PXptsS486A and Myc-PDK1 in HeLa (not demonstrated) or
COS-1 cells (Fig. 6), no peroxisomal staining was observed for
Myc-PDK-1 despite distinct peroxisomal localization of GFP-
CISKΔPXptsS486A. These observations point to a crucial role
for phosphorylation of CISK residue 486 in the interaction
between CISK and PDK-1.

To verify that the interaction between CISK and PDK-1 is
dependent on prior phosphorylation of CISK as suggested for
Akt and SGK1, we wanted to test the ability of PDK-1 to
directly phosphorylate CISK. Since CISK is not phosphorylated
in vitro, as we observe in vivo, we mutated Ser486 in the
hydrophobic motif of CISK to Asp to mimic a phosphorylated site.
Recombinant protein of MBP alone, MBP-CISK WT-(96–497)
or MBP-CISK S486D-(96–497) was incubated in the presence
or absence of PDK-1, as described under “Experimental Proce-
dures.” As shown in Fig. 7, we find that CISKS486D is phos-
phorylated by PDK1 in vitro. In contrast, the wild type CISK is
not phosphorylated, which confirms that prior phosphorylation
of the hydrophobic motif of CISK is necessary for PDK-1 inter-
action and phosphorylation.
FGF-2), vascular endothelial growth factor (VEGF), interferon proteins such as fibroblast growth factor-1 and -2 (FGF-1 and FGF-2), in a piggy-back fashion into the organelle lumen (16, 17). This was demonstrated for Myc-Col BD and GFP-ImmE2 and further increases the versatility of this protein-protein interaction assay.

We have provided evidence both by immunofluorescence microscopy and electron microscopy that the protein complexes were completely translocated into the organelle lumen and did not remain as aggregates at the surface of the organelle. It is important to ensure complete translocation to ascertain that the interacting partners bind to each other in the cytosol with a binding affinity tight enough for translocation into the organelle lumen to occur. To yield a positive result, this system does not simply require that the molecules are in close proximity to each other, like other methods such as FRET or BRET (12, 13), but that the interacting proteins are able to traverse an intracellular membrane together as a complex. As a consequence the interacting proteins are relocated to a defined subcellular compartment, which are easily defined by confocal microscopy by using the catalase as an organellar marker.

The principle of co-importing interacting proteins to a subcellular compartment by tagging one protein with a specific targeting signal is not entirely new. The group of Stephen Gould showed that by adding a nuclear localization sequence to Pex19 (Pex-NLS) this protein together with interacting peroxisomal membrane proteins were mislocated to the nucleus (27). We think that this system has several disadvantages as compared with our system. By targeting the protein complex to the nucleus it is impossible to assay for interactions involving any of the numerous intracellular proteins that at some point are present in the nucleus. In contrast to this, only the comparatively few peroxins are excluded from use by our assay. Furthermore, small proteins may diffuse through the nuclear pore complex and into the nucleus yielding false positive results. In contrast, the peroxisomal membrane is continuous and will not allow any leakage into the organelle. Most importantly, compared with other intracellular compartments such as the nucleus, the small size and the punctuate staining pattern of the peroxisomes are a great advantage when imaging co-localization and the major reason for employing this organelle as the destination for the protein complex. Additionally, the technique we implemented of merging the images askew by one peroxisomal diameter yields a clear yes or no signal for co-localization of this protein-protein interaction assay.

A requirement for the peroxisomal targeting assay is that the PTS-tagged protein is efficiently targeted to the peroxisomes. We have added the PTS tag to a range of different proteins such as fibroblast growth factor-1 and -2 (FGF-1 and FGF-2), vascular endothelial growth factor (VEGF), interferon gamma (IFNγ), ciliary neurotrophic factor (CNTF), CREB-binding protein KIX domain and the HIV-TAT protein. All constructs, apart from HIV-TAT, were efficiently recruited to the peroxisomes when expressed in the cytosol without a signal sequence. This suggests that most proteins are efficiently targeted to the organelle merely by the addition of a tripeptide at the extreme C terminus. All cell lines tested so far (U2OS, NIH/3T3, HUVE, Hep2, COS-1, and HeLa) are able to import PTS-tagged proteins into the peroxisomes. This system is therefore a highly versatile tool allowing protein-protein interaction to be studied directly in any cell of interest by merely adding a PTS tag to one of the proteins in question. This might be an indispensable requirement if the interaction to be tested is specific for certain cell lines. Additionally, the ability to assay for interactions dependent on post-translational modifications is a great advantage as compared with alternative systems using bacteria and yeast.

The presence of the PTS-1 tripeptide at the extreme C terminus will presumably not influence the binding capability of most proteins. However, when the C terminus is of functional importance, such as in proteins carrying a CAAX-box or a PDZ-binding site, the PTS-1 tag can alternatively be added to the suspected binding partner. This was demonstrated for Myc-Col BD and GFP-ImmE2 and further increases the versatility of this protein-protein interaction assay.

We have provided evidence both by immunofluorescence microscopy and electron microscopy that the protein complexes were completely translocated into the organelle lumen and did not remain as aggregates at the surface of the organelle. It is important to ensure complete translocation to ascertain that the interacting partners bind to each other in the cytosol with a binding affinity tight enough for translocation into the organelle lumen to occur. To yield a positive result, this system does not simply require that the molecules are in close proximity to each other, like other methods such as FRET or BRET (12, 13), but that the interacting proteins are able to traverse an intracellular membrane together as a complex. As a consequence the interacting proteins are relocated to a defined subcellular compartment, which are easily defined by confocal microscopy by using the catalase as an organellar marker.

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In all protein-protein interaction assaying systems, the strength of binding between the proteins in question will be a limiting factor. We have observed throughout our study that the efficiency of peroxisomal recruitment increases with the binding strength between the two proteins. Co-localization of ColBDpts and ImmE2, or ColBD and ImmE2pts, was detected in virtually all the peroxisomes stained by the peroxisomal marker catalase, while the CISKaFXpts-PDK-1 protein complex was recruited to a lesser extent. This explains why the ImmE2-ColBD images are of somewhat better quality than for proteins interacting with a lower affinity. However, it is still...
possible to yield a clear yes or no signal for binding affinities in the physiological binding range, as seen for the CISK-PDK-1 interaction. The lower limit of binding affinity detected by this assay remains to be assessed.

Similarly, an upper size limit of the protein complex to be imported into the peroxisomes also remains to be established. Oligomers as large as 443 kDa have been reported to be trans-located into the peroxisomal lumen (16), which should allow the method to be used for large and perhaps multimeric complexes. This is an advantage compared with other systems like FRET where the fluorophores must be in close proximity to each other to detect a protein-protein interaction. The probability to yield such a positive result will then decrease as the size of the protein complex increases.

The Ser/Thr kinase CISK shares regulatory features with and possesses a homologous kinase domain to other SGK family members, such as Akt and SGK1. Additionally, these kinases share the same subset of substrates, including Bad and the forkhead transcription factor FKHR1 (1). The activation of CISK is believed to be dependent on phosphorylation of two amino acid residues, Thr320 in the activation loop and Ser486 in the hydrophobic motif of the kinase domain, that are conserved in the related Ser/Thr kinases Akt and SGK1. PDK1 is known to phosphorylate Akt and SGK1 in the activation loop. Here we provide evidence for such a phosphorylation dependent interaction between CISK and PDK-1. By employing the peroxisomal targeting system we find that the interaction of CISK and PDK-1 is dependent on phosphorylation of Ser486 in the hydrophobic motif of CISK. Furthermore, we show in vitro that this residue needs to be phosphorylated to facilitate PDK-1 phosphorylation. These findings are consistent with earlier reports showing that overexpression of SGK3 Ser415 → Asp mutant in cells resulted in constitutive phosphorylation at the PDK1 site and that this mutant was a better substrate for PDK1 than the wild type in vitro (23–25). Collectively, these results therefore suggest that phosphorylation of the hydrophobic motif of CISK is important for its function by creating a surface for the interaction with PDK-1.

It has been suggested that the PX domain may be involved in protein-protein interactions and signaling (26). We can, however, in this case exclude that the PX-domain is required for CISK to bind PDK-1.

If the PTS-tagged protein contains an alternative targeting sequence, such as a membrane targeting signal or a nuclear localization signal, the protein might be directed to the alternative location rather than to the peroxisomes, which then reduces the applicability of this system. However, peroxisomal targeting could also be used to study such alternative localization signals. If deletion or mutation of amino acids suspected to...
be implicated in the signal for the alternative localization, such as lysines in putative nuclear localization signals, results in a shift toward peroxisomal localization, this suggests a crucial role for these specific residues in the primary targeting.

Relocation to the peroxisomes may also be difficult or impossible if a protein is associated with or inserted into cellular membranes. The solution to this concern is to use a truncated and then soluble version of the protein where the membrane-retaining domain is deleted. We demonstrate this in the case of CISK, which is made soluble after deleting the PX domain and then soluble version of the protein where the membrane-retaining domain is deleted. We demonstrate this in the case of CISK, which is made soluble after deleting the PX domain responsible for attaching the protein at the early endosomal membranes. In this way, studies on putative membrane attachment protein domains are feasible by exploiting peroxisomal targeting.

The peroxisomal targeting assay also has the potential for detecting unknown protein-protein interactions. A protein containing a PTS tag will bind its unidentified interaction partners in the cytoplasm and then co-transport them into the peroxisomes in a piggy-back fashion, which results in an up-concentration of the interaction partners in these organelles. After isolating the peroxisomes into a highly pure fraction, conventional identification methods such as SDS-PAGE combined with mass spectrometry can be used to identify the novel interacting partners. Such an approach was recently used to identify new peroxisomal proteins as reported by Kikuchi et al. (28).

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Peroxisomal Targeting as a Tool for Assaying Protein-Protein Interactions in the Living Cell: CYTOKINE-INDEPENDENT SURVIVAL KINASE (CISK) BINDS PDK-1 IN VIVO IN A PHOSPHORYLATION-DEPENDENT MANNER
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