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Targeting efficiencies of various permutations of the consensus C-terminal tripeptide peroxisomal targeting signal

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Two types of peptide signals are known to independently target proteins into the peroxisomal matrix. One of these is a consensus C-terminal tripeptide which is conserved in many microbody proteins derived from diverse species. The second signal is an N-terminal sequence found in a small subset of peroxisomal proteins. We have tested 18 possible variants of the consensus tripeptide targeting signal for their ability to facilitate the transport of a cytosolic passenger protein, chloramphenicol acetyltransferase, into peroxisomes of monkey kidney cells. Our results reveal the presence of a hierarchy of preferred amino acid substitutions at each position of the tripeptide.

Peroxisomal targeting signal; Protein sorting; Protein trafficking; Peroxisome biogenesis; Topogenesis of peroxisomal protein

1. INTRODUCTION

In the last few years much insight has been gained regarding the signals that target proteins into the peroxisomal matrix [1–5]. The first peroxisomal targeting signal to be elucidated in detail was the C-terminal tripeptide, Ser-Lys-Leu (SKL), of firefly luciferase which was shown to be both necessary and sufficient for peroxisomal targeting [1–3]. This tripeptide is necessary for the transport of luciferase into the peroxisomal matrix in mammalian [3] and yeast cells [6] and for the targeting of cytosolic chloramphenicol acetyltransferase (CAT) into glycosomes of *Trypanosoma brucei* [7]. Many peroxisomal, glyoxysomal and glycosomal proteins from diverse species were recognized by an antibody directed against the SKL tripeptide, suggesting that this tripeptide served as a general microbody targeting signal [8,9]. Mutational analysis of this tripeptide suggested that the Ser could be replaced by Ala or Cys, the Lys by His or Arg, but that the C-terminal amino acid had to be Leu. Because some peroxisomal proteins have now been found with a C-terminal M, we first tested whether the SKM tripeptide would serve as a PTS when fused onto the C-terminus of CAT. We also tested the other 17 permutations of the C-terminal tripeptide PTS, including the methionine substitutions, by expressing the CAT-fusion proteins in monkey kidney (CV1) cells and deducing their subcellular localizations by double indirect immunofluorescence [1].

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2. MATERIALS AND METHODS

The oligonucleotides shown in Table I were used to fuse various tripeptide permutations at the C-terminus of the CAT protein using PCR. Seventeen separate PCR reactions were performed using each of the oligos 1–17 listed in Table I as the 3' primer, the oligo OB5' as the 5' primer (binds to nucleotides 6 to 1 and nucleotides 5243–5221 of SV40) and the vector pSV2CAT [10] as the template. The PCR products were digested with *Hin*dIII and *Bgl*II and the DNA fragments containing the open reading frames for the fusion proteins were cloned downstream of the SV40 early promoter in the pSV2 vector. The CAT-SKL fusion protein was also expressed from the pSV2 vector as described earlier [3].

Each of the CAT-fusion plasmids was transfected into CV1 monkey kidney cells and double indirect immunofluorescence was used to localize the fusion proteins as described in Gould et al. [1,3]. The CAT-fusions were detected 48 hrs post-transfection using the mouse monoclonal antibody produced by the CAT2 cell line and fluorescein-conjugated goat anti-mouse antibody. Peroxisomes in these cells were identified by rabbit antibodies raised against the SKL tripeptide and rhodamine-conjugated goat anti-rabbit antibody [8]. The anti-SKL antibody appears to be specific for proteins ending in the sequence SKL ([9]; R. Rachubinski, personal communication).

3. RESULTS AND DISCUSSION

In attempting to quantitate the targeting efficiencies of various PTS combinations, we used the heterogeneity in expression levels of the CAT-fusions in different transiently transfected cells to evaluate, in a qualitative manner, the efficiency of transport into peroxisomes The PTS permutations exhibited a wide variety of tar-

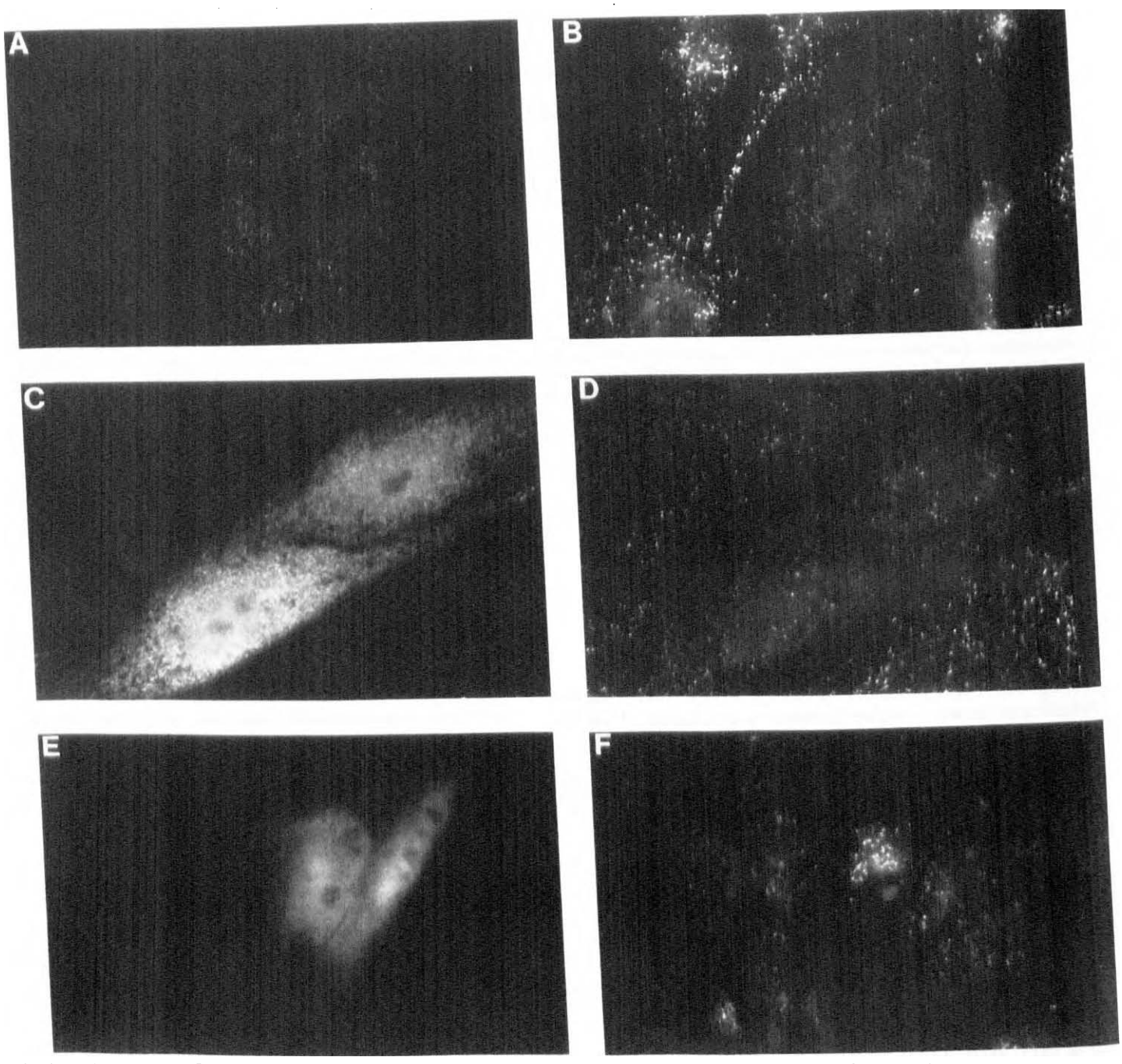

Fig. 1. Indirect immunofluorescence of monkey kidney cells showing the subcellular localization of representative examples of the three classes of CAT fusion proteins. CV1 cells were transfected with DNAs corresponding to the various CAT fusion proteins listed in Tables I and II and processed for indirect immunofluorescence 48 h later as described in Materials and Methods. The primary antibodies used were a mouse monoclonal antibody against the CAT protein (panels A, C and E) and a rabbit anti-SKL antibody directed against peroxisomal matrix proteins (panels B, D and F) [8]. Secondary antibodies were used as described in Materials and Methods. Cells were transfected with DNAS encoding the CAT-SKL (panels A, B), CAT-AKM (panels C, D) and the CAT-CHL (panels E, F) fusion proteins, respectively. These three fusion represent examples of proteins that were targeted to peroxisomes efficiently (+ in Table II), inefficiently (+/- in Table II) or not at all (− in Table II).

geting efficiencies that could be grouped into three broad classes (Table II). The first class (+ in Table II) showed as efficient targeting as the CAT-SKL fusion containing the proto-type PTS. In cells expressing fusion proteins with class 1 PTS permutations, punctate staining was observed that colocalized with the peroxisomes (Fig. 1A,B). Only in cells expressing large amounts of class 1 fusion proteins was some cytosolic fluorescence observed, suggesting saturation of the peroxisomal import system (data not shown) as has been

Table I

Oligonucleotides used for generation of CAT fusion proteins.

Oligonucleotide number	Sequence (5' to 3')	Fusion protein
1	GGA GAT CTA CAT CTT TGA CGC CCC GCC CTG CCA	CAT-SKM
2	GGA GAT CTA CAT CTT CGC CGC CCC GCC CTG CCA	CAT-AKM
3	GGA GAT CTA CAT CCG TGA CGC CCC GCC CTG CCA	CAT-SRM
4	GGA GAT CTA CAT CCG CAG CGC CCC GCC CTG CCA	CAT-ARM
5	GGA GAT CTA CAG CTT TGC CGC CCC GCC CTG CCA	CAT-AKL
6	GGA GAT CTA CAG TCG TGA CGC CCC GCC CTG CCA	CAT-SRL
7	GGA GAT CTA CAG GTG TGA CGC CCC GCC CTG CCA	CAT-SHL
8	GGA GAT CTA CAG CTT ACA CGC CCC GCC CTG CCA	CAT-CKL
9	GGA GAT CTA CAG ATG ACA CGC CCC GCC CTG CCA	CAT-CHL
10	GGA GAT CTA CAG TCG ACA CGC CCC GCC CTG CCA	CAT-CRL
11	GGA GAT CTA CAT CTT ACA CGC CCC GCC CTG CCA	CAT-CKM
12	GGA GAT CTA CAT ATG ACA CGC CCC GCC CTG CCA	CAT-CHM
13	GGA GAT CTA CAT TCG ACA CGC CCC GCC CTG CCA	CAT-CRM
14	GGA GAT CTA CAT ATG TGA CGC CCC GCC CTG CCA	CAT-SHM
15	GGA GAT CTA CAT ATG TGC CGC CCC GCC CTG CCA	CAT-AHM
16	GGA GAT CTA CAG ATG TGC CGC CCC GCC CTG CCA	CAT-AHL
17	GGA GAT CTA CAG TCG TGC CGC CCC GCC CTG CCA	CAT-ARL
OB5'	CGA GGC CGC CTC GGC CTC TGA GCT ATT CC	-

described earlier [2]. The second class (+/− in Table II) exhibited inefficient but detectable targeting into peroxisomes. In cells expressing low amounts of the class 2 fusion proteins, punctate staining, that colocalized with peroxisomes, could be seen on top of the cytosolic fluorescence. An example of this class (CAT- AKM) is shown in Fig. 1C,D. In cells expressing large amounts of the class 2 fusion proteins, the punctate staining was masked by the strong cytosolic fluorescence (data not shown). The third class of fusion proteins (− in Table II) showed no detectable peroxisomal targeting, independent of the level of expression (see CAT-CHL, Fig. 1E,F). Table II summarizes the results for all the fusion proteins tested.

The following conclusions may be drawn from these results. Not all possible permutations of the consensus tripeptide can actually function as PTSs in monkey kidney cells. It is possible that this hierarchy might be different in other organisms. For example, *Candida* sp. appear to use AKI as an efficient PTS whereas mammalian cells do not (R. Rachubinski, personal communication). In the first position, S and A function equally well but are preferred over C. In the second position, K and R function equally well but are preferred over H. In the terminal position, there is a strong preference for L over M. PTS combinations with one suboptimal residue are still functional but those with two or more suboptimal residue are not. Recent evidence from Keller et al. [9] has shown that the tripeptide PTS is likely to be used in targeting proteins to all microbodies (i.e. peroxisomes, glyoxysomes, glycosomes), and has led to the renaming of this consensus tripeptide PTS as a microbody targeting signal (McTS). However, it is not the only PTS identified to date [4,5]. An examination of the sequences of known peroxisomal sequences reveals that the functional consensus tripeptide PTS variants defined in this paper are found in at least 25 microbody proteins from diverse species [11], lending credence to the fact that the tripeptide McTS serves as a major, but not exclusive, signal for the targeting of proteins into the peroxisomal matrix.

Table II

Targeting Efficiencies of Permutations of the Tripeptide PTS

	K	R	H
S	L + M +/−	L + M +/−	L + M −
A	L + M +/−	L + M +/−	L +/− M −
C	L + M −	L + M −	L − M −

The first amino acid of the tripeptide PTS is shown on the left, the second amino acid is shown at the top and the C-terminal amino acid (L or M) is indicated in the boxes in the middle. The proteins ending in these tripeptides were transported into peroxisomes of monkey kidney cells efficiently (+), inefficiently (+/−) or not at all (−).

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