Signal peptide fragments of preprolactin and HIV-1 p-gp160 interact with calmodulin

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Secretory proteins and most membrane proteins are synthesized with a signal sequence that is usually cleaved from the nascent polypeptide during transport into the lumen of the endoplasmic reticulum. Using site-specific photo-crosslinking we have followed the fate of the signal sequence of preprolactin in a cell-free system. This signal sequence has an unusually long hydrophilic n-region containing several positively charged amino acid residues. We found that after cleavage by signal peptidase the signal sequence is in contact with lipids and subunits of the signal peptidase complex. The cleaved signal sequence is processed further and an N-terminal fragment is released into the cytosol. This signal peptide fragment was found to interact efficiently with calmodulin. Similar to preprolactin, the signal sequence of the HIV-1 envelope protein p-gp160 has the characteristic feature for calmodulin binding in its n-region. We found that a signal peptide fragment of p-gp160 was released into the cytosol and interacts with calmodulin. Our results suggest that signal peptide fragments of some cellular and viral proteins can interact with cytosolic target molecules. The functional consequences of such interactions remain to be established. However, our data suggest that signal sequences may be functionally more versatile than anticipated up to now.

Keywords: calmodulin/eukaryotic signal peptidase/HIV-1 gp160/prolactin secretion/signal sequence

Introduction

Secretory proteins and membrane proteins contain a signal sequence for targeting to the protein-conducting channel and subsequent translocation across or insertion into the endoplasmic reticulum (ER) membrane (Rapoport *et al.*, 1996). During transport into the ER lumen the signal sequence is often cleaved from the precursor protein by the signal peptidase (Blobel and Dobberstein, 1975). The characteristic feature of a signal sequence is a tripartite structure: a polar N-terminal n-region, a hydrophobic core (h-region) of 7–15 residues and a polar C-terminal c-region that contains the consensus sequence for signal peptide cleavage (von Heijne, 1985). The n-region of most signal sequences comprises only a few residues. However, some signal sequences have extended n-regions, of up to 150

residues. The function of such long n-regions is not as yet known.

The fate of only a few signal sequences has been elucidated. Fragments derived from the signal sequence of some secretory proteins or type I membrane proteins have been found associated with MHC class I molecules and are transported to the cell surface for presentation to cytolytic T cells. Some signal peptide fragments (SPFs) corresponding mainly to C-terminal segments of the respective signal sequence become associated with MHC class I molecules independent of the transporters associated with antigen processing (TAP) (Henderson *et al.*, 1992; Wei and Cresswell, 1992). However, for one SPF derived from the n-region of the lymphocytic choriomeningitis virus envelope protein a strictly TAP-dependent binding to MHC class I molecules has been reported (Hombach *et al.*, 1995). These results indicate that SPFs can be released from the membrane to the ER lumen or to the cytosol. Besides functioning in antigen presentation, nothing is known about the physiological roles of SPFs released into the cytosol or the ER lumen.

Using a synchronized *in vitro* system we have previously shown that the cleaved signal peptide of the secretory protein hormone preprolactin (p-Prl) is further processed in the ER membrane and that the resulting N-terminal SPF is released into the cytosol (Lyko *et al.*, 1995). Processing of the cleaved signal sequence was found to be sensitive to the immunosuppressive proline isomerase inhibitor cyclosporin A (Klappa *et al.*, 1996). Cyclosporin A is known to bind to cellular proteins termed cyclophilins which have proline isomerase activity and are thought to modulate the activity of various enzymes (Schreiber and Crabtree, 1992). It is thus conceivable that a cyclophilin in the ER regulates signal sequence processing and subsequent release of the SPF into the cytosol.

To determine possible functions of SPFs released into the cytosol, we followed the fate of the p-Prl signal sequence and identified components interacting with the cleaved signal sequences in the membrane and the SPF in the cytosol using site-specific photo-crosslinking (Martoglio and Dobberstein, 1996). We found that in the cytosol the p-Prl SPF interacts efficiently with calmodulin (CaM). The p-Prl signal sequence has an extended basic n-region such that it can potentially form a basic amphipathic α (baa)-helix. This feature is characteristic for CaM binding domains (O'Neil and DeGrado, 1990) but is not found in the majority of signal sequences. The HIV-1 envelope protein gp160 also has a signal sequence with an extended n-region that can potentially form a baa helix. As with p-Prl, we followed the fate of the p-gp160 signal sequence and found that a p-gp160 SPF is released into the cytosol and interacts with CaM. A synthetic p-gp160 SPF corresponding to the N-terminal 23 amino acid residues of the p-gp160 signal sequence has high affinity

Fig. 1. Signal sequence cleavage, processing and release. (A) p-Prl*/86 chains were inserted into rough microsomes (lanes 1 and 2) and subsequently released from the ribosome by addition of puromycin. Incubation was continued for 1 (lanes 3 and 4) and 15 min (lanes 5 and 6). Before application to SDS–polyacrylamide gels membranes (pellet, P) were separated from the cytosol (supernatant, S) by centrifugation. SP indicates the cleaved signal sequence, SPF the signal peptide fragment. (B) Outline of the p-Prl signal sequence and the p-Prl SPF. The h-region of the signal sequence is shaded. Italic letters in the p-Prl SPF indicate that the C-terminal end of the fragment is estimated (see Lyko *et al.*, 1995).

for CaM and efficiently inhibits Ca^{2+} /CaM-dependent phosphodiesterase *in vitro*. Our results suggest that SPFs of distinct signal sequences may interfere with CaM functions and may act as regulatory peptides.

Results

Cleavage and processing of the p-Prl signal sequence

We used a previously established system to follow the fate of the cleaved signal sequence of p-Prl (Lyko *et al.*, 1995). A truncated mRNA coding for the 86 N-terminal amino acid residues of p-Prl was translated in the presence of rough microsomal membranes. Since truncated mRNAs lack a stop codon, termination of translation does not occur. Under these conditions p-Prl/86 chains are inserted into the translocation complexes of the microsomes and remain bound to the ribosome (Gilmore *et al.*, 1991). Signal sequence cleavage does not occur because the p-Prl/86 chains are too short (Figure 1A, lane 1). To remove non-inserted p-Prl/86 chains, microsomes are then isolated, resuspended in cytosolic extracts and p-Prl/86 chains released from the ribosome by addition of puromycin. p-Prl/86 chains become translocated across the microsomal membrane and the signal sequence is cleaved. Finally, membranes are separated from the cytosol by centrifugation and both the membrane pellet and the cytosol (supernatant) are analysed by SDS–PAGE.

When p-Prl/86 chains were released from the ribosome with puromycin and incubation was continued for 1 min, the signal sequence was cleaved by the signal peptidase and was found associated with the membrane (stage I; Figure 1A, lanes 3 and 4, and B). After longer incubation (15 min) the signal sequence was cleaved and processed further by an as yet unknown signal peptide peptidase and an SPF was found in the cytosol fraction (stage II; Figure 1A, lanes 5 and 6, and B). The same result was obtained when mRNA coding for a mutant p-Prl, p-Prl*, was used which contains additional methionines at positions 12 and 13 for better labelling of the SPF with [^{35}S]methionine (Lyko *et al.*, 1995).

Membrane components interacting with the cleaved p-Prl signal sequence

In order to probe the molecular environment of the signal sequence by site-specific photo-crosslinking, the photo-activatable amino acid L-4'-(3-[trifluoromethyl]-3*H*-diazirin-3-yl)phenylalanine [(Tmd)Phe; Figure 2A] was co-translationally incorporated into the p-Prl signal sequence instead of Val18 (see Figure 1B) to give p-Prl*^T (for site-specific photo-crosslinking using (Tmd)Phe see Martoglio and Dobberstein, 1996, and references therein). p-Prl*^T/86 chains were then used for membrane insertion and puromycin release as described above.

We first probed the molecular environment of the signal sequence at stage I, when the cleaved signal sequence is still associated with the membrane (see above). Crosslinking was induced with UV light 1 min after addition of puromycin. We found two major crosslink products with apparent molecular weights of ~20 kDa and 4–5 kDa in the membrane fraction as revealed by SDS–PAGE (Figure 2B, lane 3, arrow and star). Immunoprecipitations with antibodies directed against the n-region of the p-Prl signal sequence and against prolactin (Prl) respectively showed that both crosslink products contain the cleaved signal sequence but not the Prl portion (Prl56; Figure 2B, lanes 5 and 6). Thus, the cleaved signal sequence (30 residues, ~3–4 kDa) is crosslinked to components with estimated molecular weights of ~17 and ~1 kDa.

Signal sequences are cleaved from the nascent precursor protein by the signal peptidase. We therefore assumed that the cleaved signal sequence is in contact with subunits of the pentameric signal peptidase complex (SPC) having molecular weights of 12, 18, 21, 22/23 and 25 kDa (Evans *et al.*, 1986). Using antibodies against the four smaller SPC subunits we could immunoprecipitate the ~20 kDa crosslink product with anti-SPC21 and to a minor extent also with anti-SPC18 antibodies (Figure 2B, lanes 7 and 8, arrow), but not with anti-SPC12 and anti-SPC22/23 antibodies (not shown). Sequence analysis of SPC21 and SPC18 has shown that these two subunits are putative serine proteases with homology to SEC11, an essential component of the signal peptidase complex in yeast (Böhni *et al.*, 1988; Greenburg *et al.*, 1989; Shelness and Blobel, 1990).

We have previously reported that the signal sequence of p-Prl is in contact with lipid molecules when short p-Prl chains are inserted into the protein-conducting channel and the signal sequence is still attached to the precursor protein (Martoglio *et al.*, 1995). Based on this finding and judged by the size of the small molecule (~1 kDa) crosslinked to

Fig. 2. Characterization of membrane components interacting with the cleaved p-PrI^T signal sequence. (A) Schematic illustration of the photo-activatable amino acid L-4'-(3-[trifluoromethyl]-3H-diazirin-3-yl)phenylalanine [(Tmd)Phe], which was site-specifically incorporated at position 18 of the p-PrI signal sequence (see also Figure 1B). (B) Photo-crosslinking of the cleaved p-PrI^T signal sequence to membrane components. p-PrI^T/86 chains were released from the ribosome by puromycin and incubated for 1 min at 22°C. Samples were then frozen in liquid nitrogen and subjected to UV light (lanes 2–6). Membranes (P) were then separated from the cytosol (S) by centrifugation and analysed for crosslink products (lanes 1–4) or immunoprecipitated with antibodies directed against the p-PrI SPF (lane 5), PrI (lane 6) and subunits of the signal peptidase complex (SPC21, lane 7, and SPC18, lane 8) respectively. The arrow indicates crosslinks to SPC21 and SPC18. Stars indicate the small crosslink product. (C) Identification of phospholipid as crosslink partner. Membranes were treated with phospholipase A₂ (lane 3) after UV irradiation and separation from the cytosol. Samples were immunoprecipitated with anti-p-PrI SPF antibodies. The arrow indicates crosslinks to SPC 21 and SPC18, the star crosslinks to phospholipids.

the cleaved signal sequence, we expected the 4–5 kDa crosslink product shown in Figure 2B (lanes 3 and 5, star) to be a lipid adduct. To test whether the low molecular weight crosslink partner is a phospholipid, we treated the sample after crosslinking with bee venom phospholipase A₂ (Martoglio *et al.*, 1995). Phospholipase A₂ cleaves phospholipids at position C-2 into fatty acid and lysophospholipid. Because the amount of the 4–5 kDa crosslink product (Figure 2C, lane 2, star) was significantly reduced after treatment with phospholipase (Figure 2C, lane 3), we can conclude that a phospholipid is part of the respective crosslink product and hence that the cleaved signal sequence is also in contact with lipid molecules in the ER membrane.

Interaction of the p-PrI SPF with a cytosolic protein

We next probed the molecular environment of the signal sequence at stage II (see above). At this stage the p-PrI signal sequence has been cleaved and processed and an N-terminal SPF has been released into the cytosol (Figure 1A, lanes 5 and 6; Lyko *et al.*, 1995). Crosslinking was now induced 15 min after addition of puromycin. In the sample subjected to UV light we found the SPF and a crosslink product with an apparent molecular weight of ~20 kDa in the cytosol fraction (Figure 3A, lane 4). Immunoprecipitations with antibodies directed against the n-region of the p-PrI signal sequence (Figure 3A, lanes 5 and 6) and against PrI (Figure 3A, lanes 7 and 8) showed that the cytosolic crosslink product contains the SPF but not the PrI portion. Thus, the SPF is crosslinked to a component with an estimated molecular weight of 16–18 kDa (~20 kDa minus ~3 kDa from the SPF).

The cytosol we used for the experiments shown in Figure 3 was prepared either from bovine brain (Figure 3A, lanes 1–8) or GH₃ cells, a prolactin-synthesizing rat pituitary cell line (Figure 3A, lanes 11 and 12). When cytosol was omitted, no ~20 kDa crosslink product was obtained in the 'cytosol' fraction (Figure 3A, lanes 9 and 10). We have also tested cytosol prepared from a human cell line (Mel Juso cells; Figure 3B, lanes 5 and 6) as well as wheatgerm extract (Figure 3B, lanes 7 and 8). As shown in Figure 3B, the ~20 kDa crosslink product is always found when cytosol is present. This result suggests that the p-PrI SPF interacts with a cytosolic component uniformly present in higher eukaryotes.

The p-PrI SPF interacts with calmodulin

When p-PrI^T/86 chains were released from the ribosome with EDTA instead of puromycin, the cytosolic ~20 kDa crosslink product was not found (Figure 4, lanes 1 and 2). This result suggests that release of the SPF and its binding to the cytosolic component depends on divalent cations. To test whether Ca²⁺ or Mg²⁺ is essential for binding, p-PrI^T/86 chains were released from the ribosome with puromycin in the presence of EGTA to chelate calcium ions. Again, no cytosolic crosslink product was observed (Figure 4, lanes 3 and 4). This suggests a calcium dependence of SPF binding to a cytosolic component.

The estimated molecular weight of the cytosolic component that is crosslinked with the p-PrI SPF is 16–18 kDa. Calmodulin (CaM) is a cytosolic calcium binding protein of ~17 kDa and a central regulator of

Fig. 3. The p-PrI*^T SPF is crosslinked to a component present in cytosol. (A) Photo-crosslinking of the p-PrI*^T SPF to a cytosolic protein. p-PrI*^T/86 chains were released from the ribosome by puromycin in the presence of cytosol prepared from bovine brain (lanes 1–8) or GH₃ pituitary cells (§, lanes 11 and 12). Cytosol was omitted in lanes 9 and 10. Samples were incubated for 15 min at 22°C and subjected to UV light (lanes 3–12) and membranes (P) separated from soluble components (S) by centrifugation. Samples were then analysed for crosslink products or immunoprecipitated with antibodies directed against the p-PrI SPF (lanes 5 and 6) and PrI (lanes 7 and 8) respectively. The major crosslink product is indicated by an arrow. (B) Photo-crosslinking of the p-PrI*^T SPF in cytosol prepared from various sources. p-PrI*^T/86 chains were released by puromycin as in (A) in the presence of cytosol prepared from bovine brain (bb, lanes 3 and 4), Mel Juso cells (mj, lanes 5 and 6) or wheatgerm extract (wg, lanes 7 and 8). Samples were further treated as in (A). The arrow indicates the major crosslink product in the cytosol fraction.

many kinases, phosphatases and transporters (Klee and Vanaman, 1982). To test whether the released p-PrI*^T SPF interacts with CaM, the potent CaM antagonist calmidazol was added to the crosslinking assay. In the presence of calmidazol the cytosolic ~20 kDa crosslink product was not observed (Figure 4, lanes 5 and 6), suggesting that calmidazol efficiently competes with the p-PrI SPF for CaM.

As shown above, no ~20 kDa crosslink product was obtained when cytosol was omitted (Figure 3A, lanes 9 and 10). When purified CaM (from bovine brain) and calcium were added, however, the ~20 kDa crosslink product was obtained (Figure 4, lanes 7 and 8), suggesting that the p-PrI SPF is crosslinked to CaM. The ~20 kDa crosslink product was also obtained when CaM prepared from *Dictyostelium discoideum* was added (Figure 4, lanes 9–12). CaM from *D. discoideum* was selected because a specific antiserum against this protein was available. With

Fig. 4. Identification of CaM as the crosslink partner. p-PrI*^T/86 chains were released from the ribosome by EDTA (lanes 1 and 2) or puromycin (lanes 3–12) in the presence of cytosol (lanes 1–6), EGTA (lanes 3 and 4) or the CaM antagonist calmidazol (lanes 5 and 6). Cytosol was omitted in lanes 7–12 but purified CaM from bovine brain (lanes 7 and 8) or *D. discoideum* (§, lanes 9–12) were added instead. After UV irradiation membranes (P) were separated from soluble components (S) by centrifugation and analysed for crosslink products or immunoprecipitated with antibodies directed against *D. discoideum* CaM (lanes 11 and 12). Crosslinks to CaM are indicated by an arrow.

this antiserum we could immunoprecipitate the ~20 kDa crosslink product and thus further characterize its identity (Figure 4, lanes 11 and 12).

The p-PrI SPF is less efficiently released into the cytosol when factors are present that prevent an interaction with CaM (Figure 4, lanes 1–6) or when cytosol, and hence CaM, is absent (Figure 3A, lanes 9 and 10). This suggests that the interaction with CaM may facilitate the cytosolic localization of the amphipathic p-PrI SPF, which otherwise remains preferentially in the lipid bilayer.

The efficiency of crosslinking between the p-PrI SPF in the cytosol and CaM was very high, up to 55% (estimated from Figure 3A, lanes 2 and 4), and indicates that the majority of p-PrI SPF was in contact with CaM. Because the amount of p-PrI SPF generated in our *in vitro* translation/crosslinking system is very low (20–100 fmol/20 µl reaction), the high crosslinking efficiency also indicates a high affinity of CaM for the p-PrI SPF. Furthermore, the high crosslinking efficiency is consistent with a tight interaction between the p-PrI SPF and CaM [for chemical properties of the carbene-generating (Tmd)Phe see Brunner, 1989]. Similar crosslinking efficiencies have been reported, for example, for the tight interaction between the signal sequence of a growing polypeptide chain and the 54 kDa subunit of the signal recognition particle during protein targeting (High *et al.*, 1993b; Martoglio *et al.*, 1995).

Release of a SPF of HIV-1 p-gp160 into the cytosol and interaction with calmodulin

The characteristic feature of a CaM binding domain is a stretch of 16–35 amino acid residues that can potentially form a basic amphiphilic α(baa)-helix (James *et al.*, 1995). Such a stretch is predicted for the N-terminal portion of the p-PrI signal sequence. To see whether other signal sequences may also interact with CaM, we searched the signal sequences of mammalian and viral proteins listed

Fig. 5. Photo-crosslinking of the HIV-1 p-gp160^T SPF to CaM. (A) Outline of the p-gp160 signal sequence. The shaded area indicates the h-region of the signal sequence. The N-terminal 23 residues are also illustrated in a helical wheel; hydrophobic residues are indicated as dark circles, basic residues as white circles. The arrow indicates the amino acid (G18) that was replaced with (Tmd)Phe for site-specific photo-crosslinking. (B) Release of the p-gp160 SPF into the cytosol. p-gp160/86 chains were inserted into rough microsomes (lanes 1 and 2) and subsequently released from the ribosome by addition of puromycin. Incubation was continued for 1 (lanes 3 and 4) or 15 min (lanes 5 and 6) and membranes (pellet, P) separated from the cytosol (supernatant, S) by centrifugation before SDS-PAGE. Lanes 4–6 show *in vitro* synthesized peptides corresponding to the 20, 25 and 30 N-terminal amino acid residues of p-gp160. The p-gp160 SPF released into the cytosol is indicated by a dot (lane 6). (C) Photo-crosslinking of the p-gp160^T SPF to CaM. p-gp160^T/86 chains were released from the ribosome by puromycin in the presence of cytosol prepared from Jurkat T cells (lanes 1–8, 14 and 15). Where indicated, calmidazol (lanes 5 and 6) or synthetic p-gp160 SPF corresponding to the 23 N-terminal residues of the p-gp160 signal sequence (lanes 7 and 8) was added in addition. Cytosol was omitted in lanes 9–13 and purified CaM from *D.discoideum* and Ca²⁺ were added in lanes 11–13. Samples were incubated for 15 min at 22°C and subjected to UV light (lanes 3–15) and membranes (P) separated from soluble components (S) by centrifugation. Membranes and the cytosol fraction of one sample were then treated with phospholipase A₂ (lanes 14 and 15). Samples were finally analysed for crosslink products or immunoprecipitated with antibodies directed against *D.discoideum* CaM (lane 13) respectively. Crosslinks to CaM are indicated by an arrow, crosslinks to lipids by a star.

in the SWISSPROT database for their potential to form a baa-helix. Most signal sequences are short (<20 amino acid residues) and, after cleavage by signal peptidase and processing by signal peptidase, are not expected to bind to CaM. However, we found one more signal sequence comprising 30 amino acid residues and consensus features for CaM binding. The signal sequence of the HIV-1 envelope protein p-gp160 has all the features for a CaM binding peptide (Figure 5A). The n-region of the HIV-1 envelope protein p-gp160 signal sequence can potentially form a baa-helix and contains several tryptophan residues often found in CaM binding domains (Vorherr *et al.*, 1990; James *et al.*, 1995).

With p-gp160 we performed analogous signal peptide release and crosslinking experiments as described above for p-Prl. Short p-gp160 chains (86 residues) were synthesized *in vitro* and inserted into the ER translocation sites of microsomal membranes (Figure 5B, lane 1). When p-gp160/86 chains were released from the ribosome by

addition of puromycin and membranes were separated from the cytosol after 1 and 15 min incubation, a [³⁵S]-methionine-labelled peptide with an apparent molecular weight of 2–3 kDa appeared in the cytosol (Figure 5B, lanes 4 and 6). Because p-gp160/86 chains contain methionine residues only in the signal sequence, the released labelled peptide is either the cleaved signal sequence or a fragment thereof.

To determine the approximate length of the peptide released into the cytosol, we synthesized marker peptides representing the entire p-gp160 signal sequence (30 amino acid residues) or N-terminal SPFs of 25 and 20 amino acid residues respectively. Comparative analysis of the peptides separated by SDS-PAGE revealed an estimated size of 20–25 amino acid residues, clearly smaller than the entire signal sequence (Figure 5B, lanes 6–9). This indicated that the released peptide is a fragment of the p-gp160 signal sequence and suggests that the p-gp160 signal sequence is rapidly processed. We could not detect

a peptide corresponding to the entire signal sequence, as was the case for the signal sequence of p-Prl. The predicted processing site of the p-Prl signal peptide is between the two leucine clusters (Figure 1B) of its h-region (Lyko *et al.*, 1995). Whether such a motif is required for signal sequence processing is not known. The h-region of the p-gp160 signal sequence also contains two clusters of amino acids with long hydrophobic side chains (-MLLGMLMI-) between which processing may occur (see Figure 5A).

We next probed the molecular environment of the released p-gp160 SPF using site-specific photo-crosslinking. The photo-activatable amino acid (Tmd)Phe was co-translationally incorporated into the p-gp160 signal sequence instead of Gly18 (see Figure 5A) to give p-gp160^T and p-gp160^T/86 chains which were used for membrane insertion and puromycin release as described above. Crosslinking was induced with UV light 15 min after addition of puromycin. We found the SPF and a major crosslink product with an apparent molecular weight of ~20 kDa in the cytosol fraction (Figure 5C, lane 4, arrow). This indicates that the peptide released into the cytosol contains (Tmd)Phe and hence must be derived from the p-gp160 signal sequence. The cytosol used for these experiments was prepared from Jurkat T cells. The same results were also obtained when cytosol prepared from bovine brain was used (not shown).

To test whether the p-gp160^T SPF is crosslinked to CaM, we released p-gp160^T/86 chains from the ribosome in the presence of the CaM antagonist calmidazol. Furthermore, we released p-gp160^T/86 chains when cytosol was omitted and when purified CaM and Ca²⁺ were added instead. The ~20 kDa crosslink product was not observed in the presence of calmidazol (Figure 5C, lanes 5 and 6) or when cytosol was omitted (Figure 5C, lanes 9 and 10). The ~20 kDa crosslink product was obtained, however, when calcium and purified CaM from *D.discoideum* (Figure 5C, lanes 11–12) or bovine brain (not shown) were present. In addition, we could immunoprecipitate the ~20 kDa crosslink product with antiserum against CaM from *D.discoideum* (Figure 5C, lane 13). These results indicate that the p-gp160^T SPF interacts with CaM. In analogy to p-Prl and based on the consensus for CaM binding, we expect that the released p-gp160^T SPF comprises the N-terminal part of the signal sequence.

Not all the p-gp160 SPF was released into the cytosol. After crosslinking a low molecular weight crosslink product appeared in the membrane fractions (Figure 5C, lane 3, star). This 4–5 kDa crosslink product was sensitive to phospholipase A₂, indicating that it is a phospholipid adduct (Figure 5C, lane 14). Thus, some p-gp160^T SPF remains in the membrane in contact with phospholipids.

Characterization of the p-gp160 SPF–CaM complex

CaM interacts with target proteins with affinity constants in the low nanomolar range (James *et al.*, 1995). We determined the affinity constant for formation of the p-gp160 signal peptide–CaM complex by fluorometric titration using dansylated CaM (Anderson and Malencik, 1986) and a synthetic peptide corresponding to the 23 N-terminal residues of the p-gp160 signal sequence. This peptide could efficiently prevent crosslinking between the p-gp160^T SPF and CaM in the signal peptide release and

crosslinking experiment with p-gp160^T/86 chains (Figure 5C, lanes 7 and 8). In the presence of calcium, dansyl-CaM showed a large increase in the fluorescence intensity upon binding of the peptide (Figure 6A). The K_d determined from two series of three titrations was 22 ± 5 nM and was derived from a non-linear curve fitting procedure. No fluorescence enhancement was observed when samples contained EGTA and no calcium (not shown).

We also determined the peptide–CaM interaction in an enzyme inhibition experiment. The inhibitory effect of the synthetic p-gp160 SPF on purified CaM-dependent cyclic nucleotide phosphodiesterase (PDE) was tested *in vitro* (Wallace *et al.*, 1984). The result of such an experiment is expressed as the concentration that inhibits enzymatic activity by 50% (IC₅₀) in solutions initially containing sufficient CaM to produce 50% of the maximum stimulation (~8 nM CaM; Figure 6B, upper panel) (Anderson and Malencik, 1986). For the p-gp160 SPF we determined an IC₅₀ value of ~30 nM (Figure 6B, lower panel).

We could not determine the K_d and IC₅₀ values for the p-Prl SPF–CaM complex because synthesis of the p-Prl SPF was technically not feasible due to the high number of leucine residues. However, similar high crosslinking efficiencies of p-gp160^T and p-Prl*^T SPFs with CaM indicate similar affinities for CaM.

Discussion

We have identified components that interact with the signal peptide of p-Prl in the ER membrane and with a SPF comprising the N-terminus of the p-Prl signal peptide in the cytosol. Right after cleavage by signal peptidase the p-Prl signal sequence accumulates in the membrane and was found to be in contact with lipids as well as the 18 and 21 kDa subunits of SPC. These two SPC subunits share homology with the *Escherichia coli* leader peptidase and the yeast Sec11 protein, shown to be an essential component of the yeast signal peptidase complex (Greenburg *et al.*, 1989; van Dijk *et al.*, 1992). We have previously shown that the p-Prl signal sequence before cleavage is in contact with lipids as well as components of the protein-conducting channel (TRAMP and Sec61 α) (Martoglio *et al.*, 1995). Our present data suggest that after cleavage by signal peptidase the p-Prl signal sequence moves from the translocon–lipid interface into proximity to SPC18 and SPC21, the putative catalytic subunits of SPC. However, no direct evidence for such a function has been obtained for the two SPC subunits.

Previously it has been shown that the cleaved p-Prl signal sequence is rapidly processed in the ER membrane and an N-terminal SPF is released into the cytosol (Lyko *et al.*, 1995). We show here with site-specific photo-crosslinking that the released p-Prl SPF can be efficiently crosslinked to CaM in a Ca²⁺-dependent manner. Activation of (Tmd)Phe with UV light generates a highly reactive carbene ($t_{1/2} \sim 1$ ns) which, in turn, reacts with any adjacent molecule, independent of whether it is a protein, a lipid or a water molecule (Brunner, 1989). Due to this unique property of the carbene, we can conclude from the crosslinking efficiency that in our cell-free system the released p-Prl SPF has high affinity for CaM. Whether such conditions are also found in cells synthesizing Prl remains to be established.

Fig. 6. Characterization of the p-gp160 SPF–CaM complex. (A) Fluorescence titration of dansyl-CaM with p-gp160 SPF. Dansylated CaM [160 (◆) or 260 nM (◻)] was titrated with a synthetic peptide corresponding to the 23 N-terminal residues of the p-gp160 signal sequence. The fluorescence intensity at 470 nm after excitation at 340 nm was recorded at each concentration of peptide. The fluorescence enhancement values were normalized for maximal enhancement at saturation and the values of three independent experiments were plotted against the ratio peptide:dansyl-CaM. (B) Effect of the p-gp160 SPF on CaM-dependent PDE-mediated hydrolysis of cAMP. Activator-deficient PDE was preincubated in the assay medium with increasing CaM concentrations (upper panel) or in the presence of 8 nM CaM and increasing concentrations of synthetic p-gp160 SPF (lower panel). The enzyme reaction was started by addition of cAMP. CaM-induced PDE activity is plotted against CaM concentration (upper panel), showing half maximal activation of PDE at 8 nM CaM. Inhibition of PDE activity at 8 nM CaM is plotted against p-gp160 SPF concentration (lower panel). The determined IC_{50} is ~30 nM.

CaM recognizes positively charged, amphiphilic α -helical stretches of 16–35 amino acid residues in CaM binding proteins as well as peptides mimicking such sites (O’Neil and DeGrado, 1990; James *et al.*, 1995). The p-Prl signal sequence has an extended n-region and the

p-Prl SPF can potentially form a basic amphiphilic α -helix. We also found this feature in the signal sequence of the HIV-1 envelope protein p-gp160, but not in the majority of other signal sequences. Thus, only a few signal sequences would yield SPFs that efficiently interact with CaM after proteolytic processing.

Cleavage of the p-gp160 signal sequence from p-gp160/86 was very inefficient in our cell-free system. It is reported that signal sequence cleavage of p-gp160 is inefficient *in vivo*, which may indicate an intrinsic property of p-gp160 (Li *et al.*, 1996). The limiting amount of p-gp160 SPF released into the cytosol did not allow rigorous characterization of the p-gp160 SPF, as shown for the p-Prl SPF. However, the size of the peptide, the presence of radioactive label and (Tmd)Phe as well as crosslinking to CaM indicate that the peptide released into the cytosol is a fragment of the p-gp160 signal sequence and contains features for CaM binding. The p-Prl SPF has been shown to comprise the N-terminal part of the signal sequence. By analogy with these data, it is likely that the p-gp160 SPF released into the cytosol also corresponds to the N-terminal part of the signal sequence. A synthetic peptide corresponding to the 23 N-terminal residues of the p-gp160 signal sequence shows low K_d and IC_{50} values, indicating high affinity of this SPF for CaM.

p-Prl and p-gp160 SPFs may act as CaM antagonists

What could be the functional and physiological significance of an interaction between the p-Prl and p-gp160 SPFs and CaM? In principle the SPF–CaM complex could acquire a novel function similar to activation of MHC molecules by peptides (Heemels and Ploegh, 1995). Another possibility is that SPFs function as CaM antagonists in the vicinity of the rough ER membrane. CaM-dependent processes may be inhibited when large amounts of a CaM binding SPF are generated and released into the cytosol. The obvious question for the latter role is whether the local concentration of SPFs can be high enough to impair CaM functions. CaM is a highly abundant protein and can account for 0.2–12 μg (11–700 pmol)/mg total cell protein depending on the cell type (Klee and Vanaman, 1982). The free CaM concentration in the cytosol, however, is difficult to estimate. CaM is often associated with CaM-dependent enzymes and considerable differences in the subcellular localization of CaM have been reported (Klee and Vanaman, 1982). The ER membrane is not especially rich in CaM-dependent enzymes and does not show an accumulation of CaM at its surface (D.Guerini, personal communication). Thus the CaM concentration at the ER surface may be similar to the free CaM concentration, which is thought to be in the range 10–100 μM (Klee and Vanaman, 1982; D.Guerini, personal communication).

In a stimulated rat pituitary cell line the amount of Prl synthesized within 10 min is ~60 ng (~3 pmol)/mg total cell protein (Gordeladze, 1990). Considering that the cytosol contains ~40% of the total cell protein and the protein concentration in the cytosol is 200–300 mg/ml (Alberts *et al.*, 1994), the p-Prl SPF concentration in the cytosol can rapidly reach ~2 μM (200–300 mg/ml $\times$ 100/40 $\times$ 3 pmol/mg), provided that all the p-Prl SPF is released

into the cytosol. Because the SPF is released only from the rough ER, the local SPF concentration is certainly higher and would exceed the local CaM concentration.

It is also likely that high levels of p-gp160 SPF will be generated when gp160 is expressed in HIV-1-infected cells. For example, the steady-state level of gp160 expressed in chronically infected cell lines has been shown to exceed the levels of actin, a highly abundant protein in mammalian cells (Geleziunas *et al.*, 1994). We therefore can expect micromolar concentrations of p-gp160 SPF in the cytosol and much higher local concentrations at the ER surface.

Release of SPFs from the ER membrane appears to be a regulated process. It was shown that the proline isomerase inhibitor cyclosporin A inhibits processing of the cleaved signal sequence (Klappa *et al.*, 1996). This suggests that a cyclophilin may modulate activity of the signal peptide peptidase and thereby regulate release of the SPF into the cytosol. Thus, a burst of SPFs may be released from the ER membrane, similarly to Ca^{2+} released from the ER lumen, and may transiently interfere with CaM-dependent processes.

The role of CaM in the regulation of cellular processes has been investigated in many studies. In experiments with tissue culture cells CaM antagonists were shown to inhibit CaM-dependent processes at low micromolar concentrations (White, 1985; Srinivas *et al.*, 1994; de Figueiredo and Brown, 1995). CaM antagonists used in these experiments (calmidazol, trifluoperazine, W13 and W7) have similar or several fold higher IC_{50} values for PDE (0.04–68 μM) than the synthetic p-gp160 SPF used in the present study. The experiments show that under physiological conditions CaM antagonists can severely affect CaM function at low micromolar concentration even though the estimated intracellular CaM concentration is considerably higher.

Possible functions of the p-Prl and p-gp160 SPFs

A possible function for the p-Prl SPF can be envisaged in Prl secretion. cAMP plays a central role in Prl expression and secretion in the anterior pituitary (Lamberts and MacLeod, 1990). The intracellular level of cAMP is regulated by adenylate cyclases (AC), synthesizing cAMP, and PDEs, hydrolysing cAMP. Ca^{2+} /CaM-dependent types of both enzymes have been characterized in mammalian cells. In the rat anterior pituitary, cells that predominantly produce Prl do not express Ca^{2+} /CaM-dependent ACs but Ca^{2+} /CaM-dependent PDE (Gordeladze, 1990; Paulssen *et al.*, 1994). The p-Prl SPF may thus compete with Ca^{2+} /CaM-dependent PDE for CaM and inhibit PDE. This would lead to prolonged cAMP signalling and continued stimulation of Prl secretion.

Viruses use many strategies to escape immune detection. They can interfere with antigen presentation or block signal transduction pathways. In HIV-1 infected cells several CaM-dependent processes involved in immune defence are disrupted (Miller *et al.*, 1993). One mechanism by which this may be achieved is binding of CaM to the cytoplasmic domain of gp41 protein (Miller *et al.*, 1993; Srinivas *et al.*, 1993). Additional binding of CaM by the p-gp160 SPF, as we show here, may further contribute to inactivation of CaM-dependent processes in infected T cells (Srinivas *et al.*, 1994).

Our results imply that signal sequences of distinct

secretory proteins and membrane proteins may have a function in addition to protein targeting and translocation. The signal sequences of p-Prl and p-gp160 have extended n-regions (>10 residues), in contrast to most other signal sequences. Interestingly, the gene for Prl encodes the signal sequence by two exons separating the n- and h-regions. In many cases exons encode distinct domains or functional regions of the final protein (Gilbert, 1985). The first exon of the *Prl* gene codes for 10 amino acid residues providing the hydrophilic and basic residues, an essential CaM binding feature, to the signal sequence of p-Prl (Truong *et al.*, 1984). Further studies with mutant p-Prl signal sequences will be required to show whether the amino acids encoded by exon 1 confer on this signal sequence an additional function and to exclude other explanations for this splicing junction.

Besides CaM, other cytosolic and even nuclear proteins may be targets for SPFs. SPFs may be derived from signal sequences with exceptionally long n-regions. Such signal sequences are often found on viral membrane proteins, like those from lentiviruses or LCMV (Pancino *et al.*, 1994; Hombach *et al.*, 1995). The length and high conservation of lentiviral signal sequences has already prompted speculations about alternative functions. Our results indicate an additional function for one type of signal sequence and demonstrate, in addition, a convenient way to identify targets for SPFs in general. Besides identifying new targets of SPFs, physiological studies are now required to determine the cellular effects of SPFs in their native cellular and organism contexts.

Materials and methods

Materials

General chemicals were from Merck (Darmstadt, Germany) or Sigma (München, Germany). Restriction enzymes, SP6 RNA polymerase and bee venom phospholipase A_2 were from Boehringer Mannheim (Mannheim, Germany). Activator-deficient PDE, 5'-nucleotidase and Fiske-SubbaRow reducer were from Sigma (München, Germany). Vectors pGEM3Z and pGEM4Z, as well as reticulocyte lysate, were from Promega (Heidelberg, Germany). [^{35}S]methionine was from Amersham Buchler (Braunschweig, Germany). Calmidazol and calmodulin were from Calbiochem (La Jolla, CA). Plasmid pL102 coding for p-gp160 was kindly provided by V.Bosch (DKFZ, Heidelberg, Germany). *Dictyostelium discoideum* CaM and the corresponding antiserum were generously provided by T.Soldati and B.Ulbricht (MPI, Heidelberg, Germany). The antibodies against SPC12, SPC18, SPC21 and SPC22/23 were gifts from E.Hartmann (MDC, Berlin, Germany) and C.Nicchitta (Duke University, Durham, NC).

Plasmids and transcription

Plasmid pL102 coding for p-gp160 was digested with *Sall* and *XhoI* and the insert DNA transferred into the *Sall* site of pGEM4Z under control of the SP6 promoter. The plasmid encoding p-Prl* (pGEM4Z/p-Prl*) has been described previously (Lyko *et al.*, 1995). The coding region of p-Prl* was transferred into pGEM3Z under control of the SP6 promoter to give pGEM3Z/p-Prl*. Codon 18 of the coding regions of p-Prl* and p-gp160 were replaced by the TAG codon using overlap extension PCR (Ho *et al.*, 1989) to give pGEM3Z/p-Prl*^T and pGEM4Z/p-gp160^T. To prepare mRNA coding for p-Prl*/86 and p-Prl*^T/86, the respective plasmids were linearized with *PvuII* and transcribed with SP6 RNA polymerase (Lyko *et al.*, 1995). To prepare mRNA coding for p-gp160*/86, the respective coding region was amplified by PCR and transcribed with SP6 RNA polymerase (Nilsson and von Heijne, 1993).

Translation and photo-crosslinking

Truncated transcripts encoding the 86 N-terminal amino acids of p-Prl*, p-Prl*^T and p-gp160*^T were translated for 15 min at 30°C in 12.5 μl reticulocyte lysate containing [^{35}S]methionine, suppressor tRNA, SRP

and dog pancreatic rough microsomes (Martoglio *et al.*, 1995). After translation, the salt concentration was increased to 500 mM KOAc and the samples incubated for 5 min on ice. Membranes were then separated by a 3 min centrifugation through a 150 μ l sucrose cushion at 48 000 r.p.m. and 4°C in a Beckman TLA100 rotor (Lyko *et al.*, 1995). The tube containing the membrane pellet was carefully rinsed with 200 μ l RM buffer [50 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 3 mM Mg(OAc)₂ and 2 mM dithiothreitol]. The membrane pellet was resuspended in 10 μ l cytosol and 10 μ l RM buffer. Cytosol was prepared from bovine brain (Celis, 1994), GH₃ cells, Mel Juse cells or Jurkat cells (Smythe *et al.*, 1992). Wheatgerm extract was prepared according to Erickson and Blobel (1983). Where indicated, EDTA (25 mM), EGTA (5 mM), calmidazol (20 μ M), CaM from bovine brain or *D.discoideum* (200 nM) and calcium chloride (100 μ M) or synthetic p-gp160 SPF (20 μ M) were also added. When cytosol was omitted, membranes were resuspended in 20 μ l RM buffer. Nascent chains were released by adding 0.8 μ l 100 mM puromycin and incubation at 22°C for 1 or 15 min. For crosslinking, samples were put on ice and UV irradiated (364 nm) for 2 min (Martoglio *et al.*, 1995). Membranes were separated from the cytosol by a 10 min centrifugation at 100 000 r.p.m. and 2°C in a Beckman TLA100 rotor.

Analysis of translation and crosslink products

Translation and crosslink products were analysed in 16.5% T, 6% C polyacrylamide gels according to Schagger and von Jagow (Schagger and von Jagow, 1987; Lyko *et al.*, 1995). Labelled proteins were visualized with a Fuji phosphorimager BAS1000. For immunoprecipitation, proteins were denatured in 1% SDS, solubilized in IP buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100) and incubated with the relevant antibodies (High *et al.*, 1993a). Antibodies were raised against peptides corresponding to the 14 N-terminal amino acids of the p-Prl signal sequence (anti-p-Prl SPF) and to residues 38–50 of Prl (anti-Prl). For phospholipid analysis membranes were resuspended in 20 μ l RM buffer with 5 mM CaCl₂ and treated with 10 U bee venom phospholipase A₂ for 5 min at 41°C (Martoglio *et al.*, 1995).

Fluorescence measurements

CaM binding by a synthetic peptide corresponding to the N-terminal 23 residues of the p-gp160 signal sequence was determined by fluorometry using dansyl-CaM according to published procedures (Anderson and Malencik, 1986; Vorherr *et al.*, 1990). The fluorescence emission of dansyl-CaM in the absence and presence of peptide was scanned from 400 to 550 nm after excitation at 340 nm using a Shimadzu RF-5000 spectrofluorometer. Samples (3 ml) contained 50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM CaCl₂ or 1 mM EGTA, 160 or 260 nM dansyl-CaM (Vorherr *et al.*, 1990) and increasing concentrations of peptide. The dissociation constant was calculated by a non-linear curve fitting procedure using Enzfitter software (Elsevier-Biosoft, Cambridge).

Enzyme inhibition assay

To establish a calibration curve of PDE activity in the presence of CaM, 0.004 U activator-deficient PDE were preincubated for 10 min at 30°C in 100 μ l 50 mM Tris-HCl, pH 8.0, 5 mM MgSO₄, 100 μ M CaCl₂, 0.02% Triton X-100 and various concentrations of CaM (Wallace *et al.*, 1984). The reaction was started by addition of 2 μ l 100 mM cAMP. After incubation for 30 min at 30°C the reaction was terminated by boiling the sample for 2 min at 95°C. 5'-AMP was subsequently converted to adenosine with 5'-nucleotidase and the phosphate released was measured by the method of Fiske and Subbarow (Wang and Desai, 1977). Inhibition assays were performed as above except that PDE was preincubated with 8 nM CaM (required for 50% CaM-induced PDE activity as determined from the calibration curve) and various concentrations of synthetic p-gp160 SPF.

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