We conclude that the efficiency of peptide transport mediated by a given type of TAP1–TAP2 is affected by the N- and C-terminal residues and by the residue next to the C terminus. There was a marked species/allele-specific effect only in the case of the C-terminal residue. Our results with the human, mouse and rat transporters indicate that the sequence of the transporters themselves contribute to the specificity of transport. As the allelic rat transporters used here differ only with regard to their TAP2 subunit, the different transport patterns can be attributed to TAP2. The contribution of sequence variations in TAP1 from different species remains to be analysed.

Our results show that not only do MHC class I molecules select peptides according to their binding motifs, but also there is some pre-selection by the transporters. Interestingly, human transporters and certain human class I molecules with Asp residues at positions 77 and 116, such as HLA-B*2705, HLA-A11 or HLA-A*6801 (ref. 25), select for peptides with positively charged amino acids (Lys, Arg) at the C terminus[16,17]. (Residues 77 and 116 are part of the F pocket which binds the C-terminal residue of a peptide.) In the mouse, which does not have Asp 116-containing class I sequences, such peptides are not transported efficiently, neither do they bind to class I molecules. In contrast Asp 116 is not uncommon in rat classical class I alleles[27]. In the case of RT1A^a, which has Asp at positions 77 and 116, there is a striking preference for peptides with Arg at the C terminus in a class I-peptide binding assay (L. Young, R. Brandt, C. J. M. Melief and G.W.B., manuscript in preparation). A deficiency in the supply of such peptides by rat TAP1–TAP2^u may therefore explain the prolonged residence of RT1A^a in the ER in TAP2^u cells[22], and is certainly consistent with the higher average hydrophobicity of peptides eluted from RT1A^a molecules loaded in the presence of TAP2^u compared with TAP2^a.

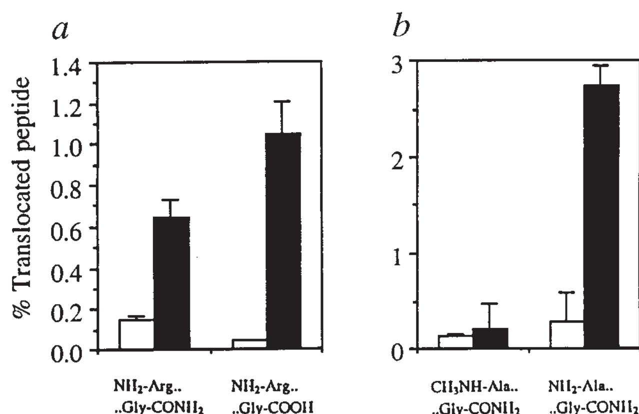

FIG. 4 *a*, Blocking of the C terminus decreases translocation. The peptides RYWANATRSG having either a free C terminus (NH_2-Arg..Gly-COOH) or a C terminus with an amide group (NH_2-Arg..Gly-$CONH_2$) were iodinated and a similar amount of radioactive input peptide was translocated in permeabilized T2 cells (open bars) and T2.rTAP1 + 2^a cells (filled bars) for 20 min. The translocated peptide is expressed as percentage of the input. Amidation reduces the translocation rate by ~40%. *b*, A free N-terminus is essential for efficient translocation of a model peptide. The peptide AYWANATRSG methylated at its N terminus (CH_3NH_2-Ala..Gly-$CONH_2$) and its non-methylated counterpart (NH_2-Ala..Gly-$CONH_2$) were used in *a*. Methylation of the N terminus drastically decreases the efficiency of peptide translocation.
METHODS. Translocation and iodination are described in Fig. 1 legend. The peptides Arg..Gly and Ala..Gly were synthesized either as free acids or acid amides by f-moc synthesis. The identity of the peptides was confirmed by mass spectrometry. The N-terminus of the peptide Ala..Gly-$CONH_2$ was methylated by reductive methylation of the t-boc deprotected peptide coupled to the resin. Peptide (260 mg) and resin were incubated at room temperature for 48 h with 1 ml acetic anhydride in 5 ml pyridine. The protective groups on the amino-acid side chains were then removed and the peptide released from the resin. Quantitative methylation was confirmed by HPLC.

The similarities in peptide translocation between rat TAP1–TAP2^u and mouse TAP1–TAP2 are also consistent with the expression of the same cytotoxic T-cell target determinants (RT1A^{a-}) when RT1A^a is expressed in mouse or rat TAP2^u cells. RT1A^a is expressed exclusively in the A^{a-} form in L cells[22] and in RMA cells (L. Young, unpublished results). It remains to be seen how much the preference of the transporters for certain peptides observed here *in vitro* reflects the transport of intracellular peptides in intact cells, which might eventually supply class I molecules with less efficiently transported peptides in amounts sufficient for antigen recognition. □

Received 8 September 1993; accepted 10 January 1994.

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ACKNOWLEDGEMENTS. We thank R. Pipkorn, R. van der Valk and L. Vernie for synthesizing peptides and T. Luts for iodinations. This work was supported by an EMBO fellowship (F.M.) and an NWO grant (J.N.)

Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1

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INTRACELLULAR signalling following mitogenic stimulation of quiescent cells involves the initiation of a phosphorylation cascade that leads to the rapid and reversible activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (refs 1, 2). MAP kinase activation is mediated by dual phosphorylation within the motif Thr-Glu-Tyr by MAP kinase kinase (MEK)[3]. Following activation, the MAP kinases translocate into the nucleus where they phosphorylate several transduction targets, including

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transcription factors[4–7]. We have previously identified PAC1 as an immediate-early mitogen-inducible tyrosine phosphatase in nuclei of T cells[8]. Here we present several lines of evidence indicating that PAC1 is a physiologically relevant MAP kinase phosphatase. Recombinant PAC1 *in vitro* is a dual-specific Thr/Tyr phosphatase with stringent substrate specificity for MAP kinase. Constitutive expression of PAC1 *in vivo* leads to inhibition of MAP kinase activity normally stimulated by epidermal growth factor, phorbol myristyl acetate, or T-cell receptor crosslinking. The inactivation of MAP kinase by PAC1 results in inhibition of MAP kinase-regulated reporter gene expression.

The catalytic domain of PAC1 (amino acids 127–314; cPAC1) was expressed as a soluble glutathione *S*-transferase (GST) fusion protein, and the enzymatic activity of the highly purified GST–cPAC1 was assayed using a variety of substrates. In a screen of potential physiological substrates, we used recombinant ERK2 that had been phosphorylated by activated MEK using [γ-^{32}P]ATP as substrate for the phosphoryl transfer. ERK2 was dephosphorylated *in vitro* by PAC1 at concentrations as low as 2 μM and did not occur with tenfold more catalytically inactive PAC1 in which Cys 257 had been replaced with serine (Fig. 1*a*). MEK activates ERK2 as a result of phosphorylation on Thr 183 and Tyr 185 (ref. 9). In some preparations of the GST–ERK2 substrate, we and others have observed phosphorylation on tyrosine and serine that was catalysed in the purified ERK2 preparation alone (Fig. 1, and ref. 10), although such phosphorylation does not activate ERK2 kinase activity. Phosphoamino acid analysis of PAC1-treated ERK2 demonstrated a loss of phosphate from threonine and tyrosine, whereas the non-MEK-catalysed phosphoserine was unaffected (Fig. 1*a*). A time course of ERK2 dephosphorylation revealed that the tyrosine residue was dephosphorylated completely before threonine. However, vanadate inhibited removal of phosphate from either amino acid (Fig. 1*a*). As expected, PAC1 treatment of activated ERK2 resulted in an inhibition of kinase activity towards myelin basic protein (MBP) (Fig. 1*b*). The reversibility of PAC1-mediated dephosphorylation was shown by the reactivation of dephosphorylated ERK2 activity following MEK treatment in the presence of vanadate (Fig. 1*b*). PAC1 phosphatase activity appears to be remarkably specific, because several other tyrosine-phosphorylated (RAYTIDE (a protein-tyrosine kinase substrate; Oncogene Science), angiotensin, casein, v-Abl) or serine/threonine-phosphorylated (casein or MEK) substrates were not dephosphorylated *in vitro* by recombinant PAC1 (Y.W. and K.K., unpublished results).

To test directly whether ERK2 is a substrate for PAC1 *in vivo*, we used a transient co-transfection assay with plasmids encoding ERK2 and either wild-type or the Ser-257 mutant of PAC1 (PAC1-S257). The levels of ERK2, PAC1 and the PAC1-S257 proteins detected in lysates from transfected cells are shown (Fig. 2*a*). Tyrosine phosphorylation of ERK2 was dependent upon epidermal growth factor(EGF)- or phorbol ester(PMA)-mediated signals (Fig. 2*b*). In activated cells, the constitutive presence of wild-type PAC1 but not of PAC1-S257 suppressed the level of tyrosine phosphorylation of ERK2 to the background level of non-transfected cells (Fig. 2*b*). In parallel with the PAC1-mediated loss of phosphotyrosine on activated ERK2, a significant loss of ERK2-catalysed MBP phosphorylation was observed (Fig. 2*c*). The MBP kinase activity assay has a lower threshold of detection for activated MAP kinase as compared to the level detectable by anti-phosphotyrosine antibodies. Extensive PAC1-catalysed dephosphorylation of ERK2 did not occur following mixing and incubation of lysates from COS 7 cells separately transfected with ERK2 and PAC1 (not shown). Also, lysates from PAC1 and PAC1-S257-transfected cells had equivalent MEK activity as assayed by an immune complex kinase assay *in vitro* (not shown), suggesting that PAC1 did not inhibit signalling events upstream of ERK2 phosphorylation.

To analyse the consequences of expressing PAC1 constitutively at moderate levels, we established permanently transfected Jurkat T-cell clones. The kinetics of ERK2 activation were investigated relative to the levels of PAC1 in parental Jurkat cells and in the PAC1-transfected Jurkat clone, G6 (Fig. 3). In unstimulated cells, the 32K PAC1 protein was not detectable in Jurkat, but a low steady-state level of PAC1 was observed in G6 cells (Fig. 3*a*). Following stimulation of Jurkat, PAC1 was observed as early as 30 min and continued to increase up to 60 min, whereas maximal levels of PAC1 were reached in G6 by 30 min after stimulation. In addition to PAC1, we observed a crossreactive, mitogen-inducible 40K protein (indicated by an asterisk in Fig. 3*a*) in Jurkat and G6 that was not recognized by antibodies to CL100, a previously described 39K protein phosphatase[11]. ERK2 activation was followed by determining kinase activity toward MBP at various times after stimulating

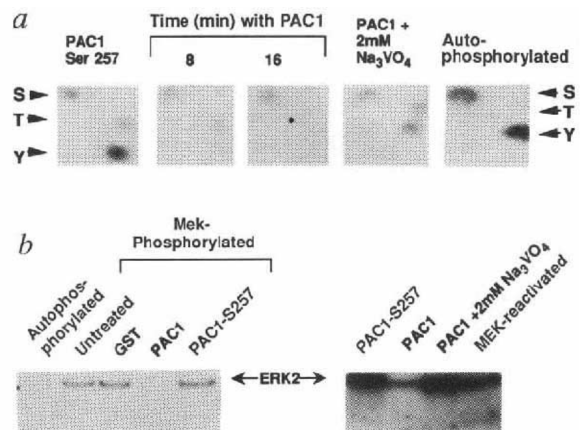

FIG. 1 PAC1 is a dual-specific Thr/Tyr phosphatase for ERK2. *a*, Phosphoamino acid analysis of autophosphorylated ERK2 and ^{32}P-GST–ERK2 incubated with GST–cPAC1 (hPAC amino acids 127–314) in the absence or presence of vanadate (2 mM) or with GST–cPAC1-S257. S, serine; T, threonine; Y, tyrosine. *b*, *In situ* protein kinase activity was determined for MEK-activated GST–ERK2 that was untreated, treated with GST–cPAC1 (±2 mM vanadate), or with GST–cPAC1-S257, or reactivated with immunoprecipitated MEK.

METHODS. *a*, After serum starvation and stimulation with phorbol myristyl acetate (PMA), confluent CV-1 cells were lysed in 25 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 25 mM β-glycerophosphate, 1 mM PMSF and 10 μg ml^{-1} leupeptin. Cell extracts were incubated for 2 h with protein A–agarose (Gibco-BRL) which had been prebound to antibody against the 16 N-terminal amino acids of MEK. GST–ERK2 (1 μg) activation was carried out by incubating for 10 min with immunoprecipitated MEK in 50 μl kinase buffer (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (10 μCi nmol^{-1})). Phosphorylated MAP kinase (0.06 μM) was incubated with either 20 μM GST–cPAC1-S257 for 16 min or 20 μM GST–cPAC1 for various time intervals in the absence of phosphatase inhibitor or for 10 min in the presence of 2 mM sodium vanadate. Dephosphorylation reactions were done in 25 μl 50 mM HEPES, pH 7.5, and 5 mM dithiothreitol at 30 °C. Although complete dephosphorylation of GST–ERK2 occurred in 1 h with GST–PAC1 concentrations as low as 2 μM, higher levels were used for shorter time intervals. Proteins were resolved by PAGE[20], electroblotted onto PVDF membrane and analysed by autoradiography. Phosphorylated amino acids were identified by phosphoamino acid analysis using two-dimensional electrophoresis on thin-layer chromatography plates[21]. Autophosphorylated MAP kinase was prepared by incubating recombinant GST–ERK2 (1 μg) in 50 μl kinase buffer for 1 h. *b*, Autophosphorylation and MEK-activation of GST–ERK2 were done as already described, except that 50 μM ATP was used. MEK-phosphorylated GST–ERK2 (0.06 μM) was untreated or incubated for 30 min with 20 μM GST alone, GST–cPAC1 or GST–cPAC1-S257. Autophosphorylated GST–ERK2 was used as a negative control for MBP phosphorylating activity (left). MEK-activated MAP kinase was incubated with GST–cPAC1-S257 or with GST–cPAC1 (±2 mM vanadate) for 10 min. Following dephosphorylation in the absence of vanadate, GST–ERK2 was reactivated in the presence of 2 mM vanadate by immunoprecipitated MEK (right). After PAGE, *in situ* MAP kinase activity for MBP was determined[22].

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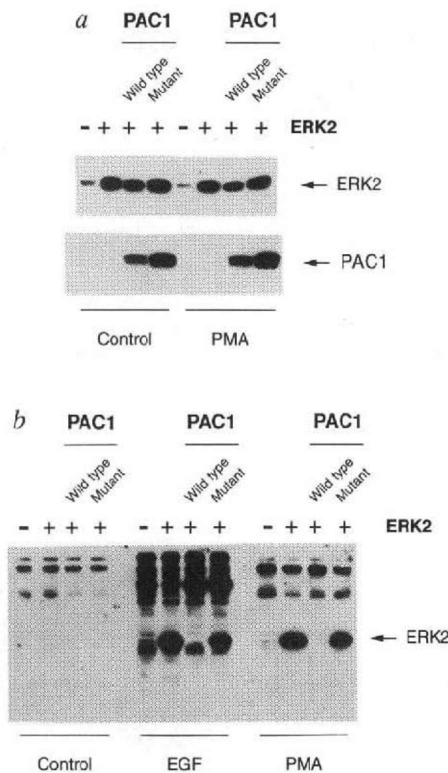
Jurkat and G6 cells (Fig. 3*b*). After mitogenic stimulation of Jurkat, ERK2 kinase activity was observed within 5 min and decreased between 30 and 60 min, coinciding with the increased expression of PAC1. By contrast, G6 cells demonstrated transient ERK2 kinase activity that was detected at 5 min but not at 30 or 60 min. The level of ERK2 activity in G6 cells at 5 min after stimulation was ~20–40% the level in comparable Jurkat cells. Relative to Jurkat cells, G6 showed dramatically reduced amounts of phosphotyrosine on ERK2 following stimulation, although approximately equivalent amounts of ERK2 were present in both cell lines (not shown). These results demonstrated that the loss of activated ERK2 correlates with the accumula-



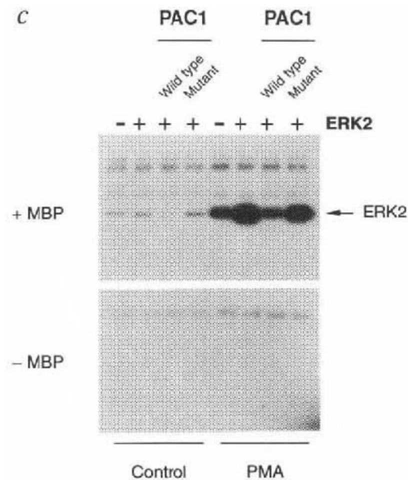


FIG. 2 MAP kinase activation is suppressed by expression of the PAC1 phosphatase. Catalytically active and inactive forms of PAC1 were co-expressed with the MAP kinase ERK2 in COS-7 cells. Cells were untreated or treated with 5 nM EGF or 10 nM PMA for 5 minutes before collecting. *a*, The level of ERK2 and PAC1 expression in untreated and PMA-treated COS cells was investigated by western blot analysis. *b*, Tyrosine phosphorylation in extracts from untreated, EGF- or PMA-treated COS cells was examined by western blotting. *c*, Map kinase activity in extracts from untreated and PMA-treated COS cells was measured using an *in situ* renaturation assay with the substrate myelin basic protein (MBP).

METHODS. Transient transfections of COS-7 cells were done as described[23]. Cells were co-transfected with 2 μg of the human ERK2 expression plasmid (pCMV-p41^{mapk}) and 2 μg of a PAC1 expression plasmid (PMT2T-PAC1 or PMT2T-PAC1-S257)[8]. Total DNA in the transfection was maintained at 10 μg using pUC13 as carrier DNA. Cells were collected 48 h after transfection. Western blot analysis was done using anti-MAP kinase monoclonal antibody 107 (R.J.D., unpublished results) and the anti-phosphotyrosine antibody PY20 (ICN) with enhanced chemiluminescence detection (Amersham). MAP kinase activity was detected after polyacrylamide gel electrophoresis using an *in situ* protein kinase assay in the absence and presence of the substrate MBP[24].

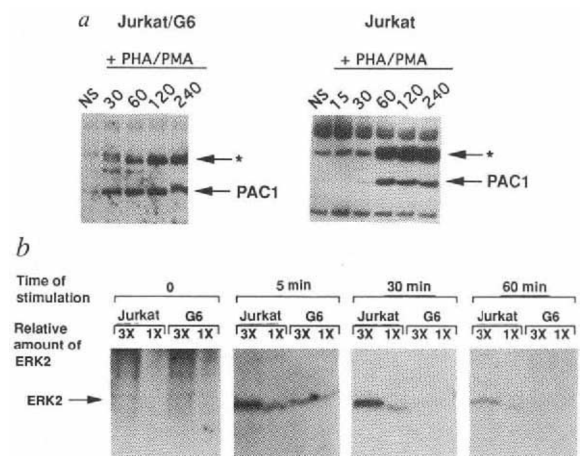


FIG. 3 Endogenous MAP kinase inactivation correlates with PAC1 expression in Jurkat T cells. Jurkat cells or a clone of Jurkat (Jurkat/G6) permanently transfected with an expression vector containing a spleen focus-forming virus LTR regulating the expression of a human PAC1 cDNA, were stimulated for various times with 1 μg ml^{-1} phytohaemagglutinin (PHA) and 10 ng ml^{-1} PMA. Cellular extracts were analysed for PAC1 expression and ERK2 activation. *a*, Steady-state PAC1 levels were measured by western blot analysis of immunoprecipitated PAC1. Stimulation times (in min) are shown above each lane. NS, not stimulated. Asterisked arrows show the 40K band discussed in the text. *b*, MAP kinase activity of immunoprecipitated ERK2 from cellular extracts was measured using an *in situ* assay with MBP as substrate.

METHODS. *a*, At various times after activation, Jurkat or G6 cells (5×10^6) were lysed with RIPA buffer[25]. Extracts were incubated overnight with three monoclonal antibodies against PAC1: P9D10, P12B11 and P10C5 (P.N.J. and K.K., unpublished) and protein G–Sepharose (Gibco-BRL). Washed immunoprecipitates were subjected to SDS–PAGE and western blot analysis with rabbit anti-PAC1 peptide (279–291) antibody[8] and ECL (Amersham). Bands above and below the arrows are crossreactions to murine IgG. *b*, At various times after activation, Jurkat or G6 cells were lysed with RIPA buffer[25]. Protein (500 μg) from each sample was denatured in 0.5% SDS for 3 min at 90 °C, diluted to 0.1% SDS with RIPA buffer, and immunoprecipitated with monoclonal anti-MAP kinase antibody (Z033) coupled to agarose (Zymed). Washed immunoprecipitates were eluted in SDS sample buffer and divided into four. To test for linearity in the assay, 3 parts and 1 part from each sample were separately resolved on SDS–PAGE containing MBP. *In situ* kinase activity was determined as described[22].

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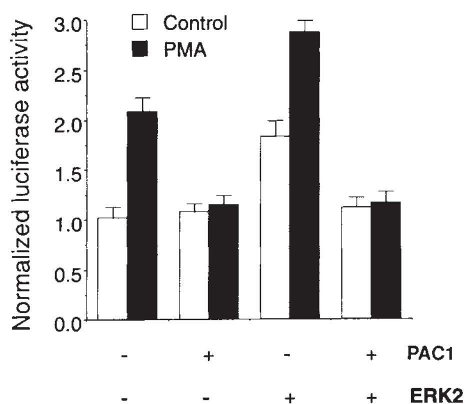

FIG. 4 PAC1 inhibits a MAP kinase signal transduction pathway leading to increased gene expression. The effect of PAC1 on the SRE-mediated expression of a luciferase reporter gene was examined using a transient transfection assay. The reporter plasmid pfos-Tk-Luc contains *c-fos* enhancer sequences including the SRE element (nucleotides −357 to −276) cloned upstream of a fragment of the thymidine kinase promoter (nucleotides −200 to 70) and the firefly luciferase gene.
METHODS. Transient transfections of CV-1 cells were done as described[23]. Cells were transfected with 2 μg of a luciferase reporter plasmid (pfos-Tk-Luc), 1 μg of a control plasmid (pCH110) expressing β-galactosidase activity, 1 μg of the ERK2 expression plasmid pCMV-p41^{mapk}, and 1 μg of the PAC1 expression plasmid pMT2T-PAC1 (ref. 8). Total DNA in the transfection was maintained at 10 μg using pUC13 as carrier DNA. Some cells (black bars) were stimulated with 10 ng ml^{-1} PMA. The cells were collected 48 h after transfection and the extracts obtained were assayed for β-galatosidase and luciferase activity[23]. The data are presented as the activity ratio of luciferase (light units per mg protein) and β-galactosidase (absorbance units per min per mg protein) (mean ± s.d., $n = 3$).

tion of endogenous PAC1 protein in Jurkat, and that ERK2 phosphorylation and activity in G6 cells are modulated by levels of PAC1 that are substantially lower than those of transiently transfected COS cells.

PAC1 is a nuclear phosphatase that is likely to act upon MAP kinases that are translocated into the nucleus following activation[6-8]. To examine the effect of PAC1 expression on a nuclear event linked *in vivo* to MAP kinase activation, we used a transient transfection assay that measures the transcriptional activity of the c-Fos serum response element (SRE). In transiently transfected CV-1 cells, SRE-regulated reporter gene expression increased ~2-fold following PMA treatment. This PMA-stimulated expression was eliminated in the presence of constitutive PAC1 (Fig. 4). Transfection with ERK2 increased the basal SRE-mediated reporter gene expression, and this was augmented by PMA treatment. PAC1 coexpression eliminated the increased basal and PMA-stimulated SRE activity resulting from ERK2 expression. These results show that increases in SRE activity resulting from PMA treatment or from increased ERK2 expression and activation are inhibited *in vivo* by PAC1. Although the specific nuclear events that act upon the SRE are not directly addressed by these experiments, a PMA-stimulated, MAP kinase-mediated pathway involving the SRF/Elk-1 ternary complex has been described[12-15].

A mitogen-induced phosphatase in fibroblasts, 3CH134/CL100, has been described[11,16]. 3CH134 has ~80% similarity with the carboxy-terminal 15K catalytic domain of PAC1, whereas the amino-terminal region of each protein (corresponding to potential regulatory domains) share ~30% similarity[11,16]. PAC1 is maximally expressed in haematopoietic tissues, whereas 3CH134 is expressed predominantly in several other tissues[8,16]. 3CH134 has been shown to dephosphorylate ERK1 and ERK2 *in vitro*[10,17,18] and to dephosphorylate ERK2 in transiently transfected COS cells[19]. The presence of more than one ERK phosphatase that can be distinctly regulated represents a means by which pleiotropic kinase activation could be differentially modulated. □

Received 26 October; accepted 29 December 1993.

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ACKNOWLEDGEMENTS. Y.W. and S.G. contributed equally to this study. The excellent technical support of Debra Campbell and Paula Davis-Searles is gratefully acknowledged. This study was supported by grants from the USPHS to R.J.D. R.J.D. is an investigator of the Howard Hughes Medical Institute.

Evolutionary conservation of components of the protein translocation complex

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PROTEIN translocation into the mammalian endoplasmic reticulum requires the Sec61p complex, which consists of three membrane proteins[1]. The α-subunit, the homologue of Sec61p of yeast[2-4], shows some similarity to SecYp[5], a key component of the protein export apparatus of bacteria[6,7]. In *Escherichia coli*, SecYp is also associated with two other proteins (SecEp and band-1 protein)[8,9]. We have now determined the sequences of the β- and γ-subunits of the mammalian Sec61p complex. Sec61-γ is homologous to SSS1p, a suppressor of *sec61* mutants in *Saccharomyces cerevisiae*, and can functionally replace it in yeast cells. Moreover, Sec61-γ and SSSlp are structurally related to SecEp of *E. coli* and to putative homologues in various other bacteria. At least two subunits of the Sec61/SecYp complex therefore seem to be key components of the protein translocation apparatus in all classes of organisms.

The Sec61p complex was purified from canine pancreatic microsomes[1]. Partial amino-acid sequences were determined for

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