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Import of a mutant mitochondrial precursor fails to respond to stimulation by a cytosolic factor

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Import of the precursor to ornithine carbamyltransferase is stimulated by a partially-purified, NEM-sensitive soluble factor from rabbit reticulocyte lysate. A mutant in which the carboxy-terminal 73 amino acids were deleted, had a sharply reduced response to this factor. The NEM-sensitive, import-stimulating factor interacts with the surface of mitochondria in the absence of precursor protein. Thus reticulocyte lysate contains an NEM-sensitive, import stimulating factor which interacts both with the surface of mitochondria and whose activity appears to be dependent upon the structure of the mature portion of the precursor.

Mitochondrial biogenesis; Cytosolic import factor; Preornithine carbamyltransferase

1. INTRODUCTION

Most mitochondrial proteins are nuclear-encoded, translated in the cytosol, and translocated across membranes to their appropriate position within the mitochondrial subcompartments. Although this process is well characterized [1–3], the machinery involved is not. In addition to the mitochondrial receptor/translocation apparatus and processing proteases, there is evidence suggesting that soluble cytosolic proteins are required as well [4–11]. It may be that an import-competent conformation effected by soluble proteins results in the enhanced translocation of proteins across both microsomal [11–13], mitochondrial [11] and bacterial cell membranes [14,15]. A recent report has shown that at least two independent soluble activities (hsp 70 and an NEM-sensitive factor) are important for efficient import of mitochondrial precursors [16].

In this report, we describe a soluble, NEM-inhibited activity in reticulocyte lysate that stimulates in vitro import of preornithine carbamyltransferase (pOCT), an activity that appears to operate independently of hsp 70. We provide evidence that this component may interact directly with a limited number of sites on mitochondria. Furthermore, by altering the structure of the mature region of the precursor protein, the import of this precursor is no longer stimulated by factor.

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Abbreviations: CCCP, carbonylcyanide-*m*-chlophenyl hydrazone; HSF, heat-stable factor; NEM, *N*-ethylmaleimide; pOCT, precursor to ornithine carbamyltransferase; pO-DHFR, dihydrofolate reductase fused to the signal sequence of pOCT

2. EXPERIMENTAL

Rat heart mitochondria were isolated and import was measured as previously described [4], except that mitochondria were washed with buffered 0.5 M KCl and 250 mM sucrose, prior to import assays. pOCT DNA cloned in pSP64 was transcribed and the resultant RNA was translated in reticulocyte lysate [17], after which cytosolic import factors were rendered limiting by either dilution of translation products or by passing them over Sephadex G-25. When rendered limiting by dilution (figs 1,3,4), 1.0 μl of translated pOCT in lysate was added to 1.0 ml import medium containing 150 μg mitochondria; and when Sephadex G-25 depleted (fig.2), 2.0 μl of the Sephadex-excluded fraction was added to 40 μl of import medium containing 20 μg mitochondria. For preparation of

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heat-stable fraction (HSF) and hsp 70, nonnuclease-treated lysate [18] was centrifuged for 2 h at 220000 × *g* and the high speed supernatant (HSS) recovered. The HSS was dialyzed against 15 mM potassium phosphate, 1 mM DTT, pH 7.0, and passed over DEAE-cellulose (0.3 ml per 1.0 ml HSS, DE-52, Whatman). The unbound fraction and wash were combined. For HSF preparation, this fraction was heated at 70°C for 15 min, and precipitated protein was removed by centrifugation. The hsp 70 preparation was obtained from the DEAE-bound fraction, and purified on ATP-agarose (essentially as in [12]).

3. RESULTS AND DISCUSSION

In the presence of the partially-purified (approx. 1000-fold) HSF, import was proportional to the amount of HSF added into the import mixture, and approached saturation at an input level of HSF equal to approx. 5 μg protein per ml (fig.1A). HSF appeared to stimulate import of pOCT by increasing both the initial rate of import and the final extent of import (fig.1B).

When HSF was pre-incubated with mitochondria in the absence of precursor and additional lysate components (fig.2), pOCT was subsequently imported with greater efficiency compared to control mitochondria (cf. lane 1 with lane 2). Further, a 0.5 M salt wash prior to import did not affect the relative stimulation (not shown), suggesting a relatively stable interaction of HSF with mitochondria. The enhancement of import efficiency was prevented if HSF was first pretreated with NEM (fig.2, lane 3), and a similar result was obtained when total lysate was substituted for HSF (fig.2, lane 6). Furthermore, when HSF or lysate were treated with NEM, import was decreased below the level of controls suggesting that NEM-inactivated HSF could still interact with the mitochondria, but in an inhibitory manner. This competitive inhibition of import by the NEM-reacted component implies the presence of specific binding sites (receptors) for HSF. That HSF was contributing to efficient import via a mechanism independent of membrane potential generation was indicated by the absence of any effect of NEM-treated HSF (or lysate) on mitochondrial membrane potential (not shown).

Recently, it has been reported that an enzymatic unfolding activity is necessary for the denaturation of native proteins that are to be translocated across microsomal [11,12] and mitochondrial [11,19] membranes. This activity has been attributed to a

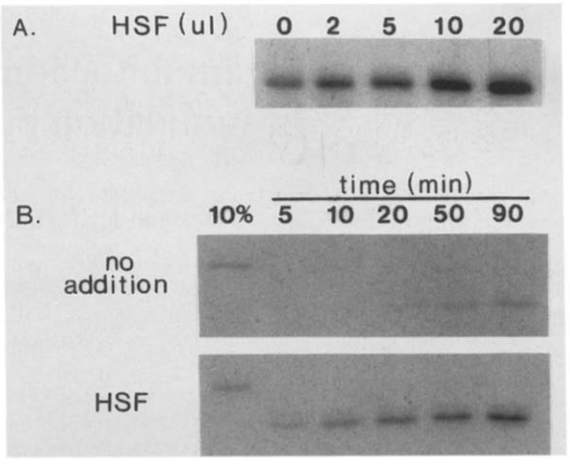

Fig.1. A soluble heat-stable fraction (HSF), partially purified from rabbit reticulocyte lysate stimulates import in a dose-dependent manner and increases the initial rate of import. (A) Dose-dependence. Increasing amounts of HSF (0.24 μg protein/μl) were added to the import assay mix. Subsequent to import (60 min at 30°C), mitochondria were diluted to 0.1 mg/ml and treated with proteinase K [4]. (B) HSF stimulates both the initial rate and the net extent of import. Either HSF (20 μl as in A) or buffer (no addition) were added to the import mixture and after designated times import was terminated by collapsing the membrane potential with 1 μM CCCP. Import and protease treatments as in A. 10% of input precursor is shown for comparison.

member(s) of the 70 kDa heat shock protein family (hsp 70). An hsp 70-enriched fraction stimulated the import of pOCT into mitochondria (lane 3, fig.3). At concentrations where HSF stimulation was saturated, stimulatory amounts of hsp 70 were apparently additive (lane 4, fig.3). This and the fact that, unlike hsp 70, HSF does not bind to an ATP-agarose column (not shown) suggest that the HSF and hsp 70 activities are distinct.

The import of a carboxy-terminal, truncation mutant, $pOCT_{282}$ was compared to the normal precursor, pOCT. The truncation mutant is reduced by approximately 20% in mass compared to full length pOCT and lacks the following C-terminal amino acid sequence:

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282                                    318
*                                      *
QGYQVTMKTAKVAASDWTFLHCLPRKPEEVDDEVFYS-

                                       354
                                       *
PRSLVFPEAENRKWTIMAVMVSLLTDYSPVLQKPKF
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Although this truncation mutant was imported

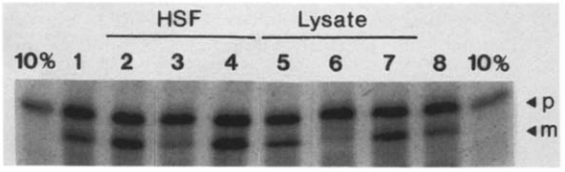

Fig.2. Pretreatment of mitochondria with HSF (or lysate) can potentiate efficient import and this potentiation can be prevented by NEM. HSF or lysate were treated with 1.5 mM NEM (lanes 3,6) or mock NEM (lanes 4,7) for 15 min at 30°C. NEM treatment was terminated by the addition of 2 mM DTT, while for mock treatment NEM was inactivated with DTT prior to reaction with HSF or lysate. Mitochondria were then incubated for 15 min at 10°C with import buffer (lanes 1,8) or with buffer containing treated HSF (lanes 2–4) or lysate (lanes 5–7). All mitochondria were recovered by centrifugation, and resuspended into import assay medium and the assay conducted as described in fig.1, except that the mitochondria were not protease-treated after import. 'p' is precursor (39 kDa), 'm' is mature imported OCT (36 kDa).

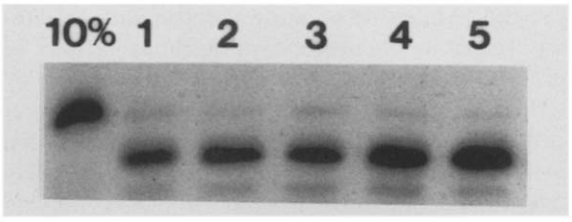

Fig.3. Import in the presence of HSF is further stimulated by the addition of an hsp 70 containing fraction. Import assays were conducted as in fig.1. Lanes: 1, no addition; 2, 30 μl HSF (7.2 μg protein); 3, 60 μl hsp 70 fraction (5.9 μg protein); 4, 30 μl HSF plus 60 μl hsp 70; 5, 10 μl (3.0 mg protein) unfractionated lysate. 10% of input precursor for comparison.

in the absence of HSF with similar efficiency as the full length pOCT (fig.4), it was not stimulated by the addition of HSF. Thus, it appears that a C-terminal domain, either directly or indirectly facilitates stimulation by factor. Furthermore, another truncation mutant (in which only amino acid residues 318–354 were deleted, see above sequence) was still stimulated by HSF (not shown), suggesting that the region important for stimulation of pOCT import by cytosolic factors was in the region encompassed by residues 282–318.

To determine if HSF stimulation was a unique property of the mature portion of pOCT, the fusion protein (pO-DHFR) containing the N-terminal signal sequence of pOCT and mouse cytosolic DHFR was tested for HSF stimulation (fig.4). Although the leader signal sequence from pOCT would be expected to accurately and efficiently target this hybrid precursor to the mitochondria [17], this precursor would not be expected to contain a sequence specific for mitochondrial import within the mature portion (normally a soluble, cytosolic protein) of the precursor. However, pO-DHFR was also stimulated by HSF, suggesting that HSF-stimulation may be due to an interaction with particular conformational determinants rather than with specific sequences.

It is apparent that at least two cytosolic activities may contribute towards the efficient import of nuclear-encoded mitochondrial precursors. Hsp 70, previously shown to stimulate translocation of proteins into microsomal membranes in vitro [11,12], and more recently mitochondrial precursors [16], also stimulates in vitro import of the mitochondrial precursor, pOCT. Another import stimulating activity, HSF, is independent of hsp 70, is relatively heat-stable, and is sensitive both to proteases (not shown) and to the sulfhydryl alkylating reagent, *N*-ethylmaleimide. This component can interact with a limited number of binding sites on mitochondria in the absence of either precursor or other lysate components. Furthermore, it can interact in a relatively stable manner with mitochondria, even in an NEM-inactivated form (fig.3), where it becomes an inhibitor of import. Together these results sug-

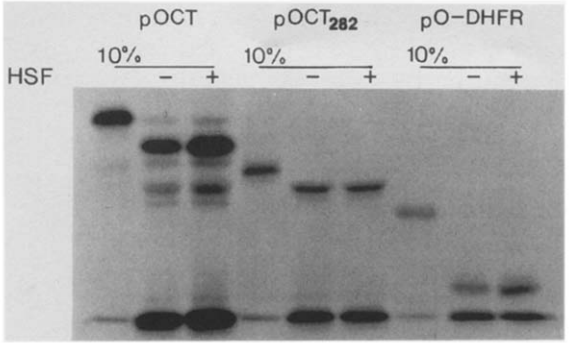

Fig.4. HSF stimulation is dependent on the structure of the mature portion of pOCT. Import assays were conducted as in fig.1. pOCT, C-terminally truncated pOCT ($pOCT_{282}$), or pO-DHFR were imported in the presence or absence of HSF. 10% of each precursor input are shown for comparison. $pOCT_{282}$ was constructed by linearizing pSP019 (see [17]) with the restriction enzyme *Bst*EII prior to transcription. pO-DHFR was constructed by fusing the N-terminal signal from pOCT (amino acids 1–32) to mouse DHFR (starting at amino acid 3) (Sheffield, W.P. and Shore, G.C., unpublished).

gest that while one or more determinants of HSF may recognize the mitochondria, another may interact with the mature portion of the precursor, perhaps retaining it in an import-competent conformation. Such observations suggest the involvement of a multi-component system. The C-terminal truncation mutant of pOCT may no longer require the latter activity if it is already import-competent. Thus, HSF may function in a manner analogous to the bacterial 'trigger factor' which can independently interact with both the bacterial plasma membrane and precursor proteins [14]. The NEM-sensitive, heat-stable factor probably does not directly employ ATP either as a substrate or cofactor for its activity as it fails to bind to an ATP-agarose column (not shown).

Recently, it has been reported that NEM-sensitive import factors are present both in the cytosol and on microsomal membranes [20]. Our results are consistent with an NEM-sensitive import component existing in either a soluble or a mitochondrial membrane-bound form.

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