A Cryptic Targeting Signal Induces Isoform-specific Localization of p46Shc to Mitochondria*

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The human Src homology and collagen (Shc) gene encodes three protein isoforms of 46, 52, and 66 kDa that belong to a family of molecular adapters involved in several signal transduction pathways. Recently, the 66-kDa isoform has been shown to play a central role in controlling reactive oxygen species metabolism and life span in mammals. Despite the large amount of information available on the biology and biochemistry of Shc proteins, very little is known regarding the regulation of their subcellular localization. Here we demonstrate the specific and selective localization of p46Shc to the mitochondrial matrix. Through deletion mapping experiments, we show that targeting of p46Shc to mitochondria is mediated by its first 32 amino acids, which behave as a bona fide mitochondrial targeting sequence. We further demonstrate that the N-terminal location of the signal peptide is critical for its function. This accounts for the observation that p52Shc and p66Shc, containing the same sequence but more internally located, display a remarkably different subcellular localization. These findings indicate that p46Shc may exert a nonredundant biological function in signal transduction pathways involving mitochondria.

Of the three Src homology and collagen (Shc) isoforms, p52Shc and p46Shc are translated from the same mRNA using two different initiation codons and are almost ubiquitously expressed, whereas p66Shc is generated from a distinct mRNA whose transcription is driven by an alternative promoter that is silenced in a tissue-specific manner (1, 2). Shc proteins share a common modular organization (an amino-terminal phosphotyrosine binding (PTB) domain, a central collagen homology region (CH1), rich in proline and glycine, and a carboxyl-terminal Src homology 2 (SH2) domain) and differ solely at their N termini. Despite this structural similarity, there is emerging evidence that the three isoforms are not functionally redundant and that p52Shc and p66Shc are implicated in distinct signal transduction pathways. Based on the finding that, following growth factor stimulation, p52Shc is recruited to the activated receptor, phosphorylated on tyrosine residues, and associates with the Grb2-SOS complex, a model has been proposed wherein this Shc isoform participates in receptor tyrosine kinase-dependent Ras activation. Consistently, overexpression of p52Shc results in (i) transformation of immortalized mouse fibroblasts (3) in a way that requires association with Grb2 (4), (ii) induction of neuronal differentiation of PC12 cells in a Ras-dependent manner (5), and (iii) enhancement of mitogen-activated protein kinase activation in response to epidermal growth factor (5) and granulocyte-macrophage colony-stimulating factor stimulation (6). On the contrary, p66Shc, which is also phosphorylated on tyrosine residues and binds Grb2 upon growth factor stimulation, is not able to activate Ras, does not induce mitogen-activated protein kinase phosphorylation, and does not transform immortalized fibroblasts (2, 7), suggesting its involvement in different pathways. Indeed, it has been recently demonstrated that p66Shc regulates intracellular levels of reactive oxygen species, reactive oxygen species-dependent apoptosis, and life span in mammals (8, 9).

Subcellular compartmentalization is an essential aspect of signal transduction, and, although Shc proteins have been reported to reside in the cytoplasm and to redistribute to the plasma membrane after growth factor stimulation (10), nothing is known regarding the specific subcellular localization of the three Shc isoforms. Here we report that p46Shc localizes to the mitochondrial matrix via a mitochondrial targeting signal that is inactive in p52 and p66Shc. These findings demonstrate a further degree of complexity in the regulation of Shc isoforms and suggest a nonredundant function for p46Shc in signal transduction pathways involving the mitochondria.

EXPERIMENTAL PROCEDURES

Cell Culture—CV1, NIH3T3, and MEFs (wild type and Shc−/−) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine calf serum, 100 μg/ml streptomycin, 100 μg/ml penicillin. Wild type MEFs were prepared according to standard procedures. Shc−/− MEFs were a kind gift from Tony Pawson. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

Antibodies—Shc antibody was obtained from Transduction Laboratories (anti-Shc SH2 monoclonal). Anti-FLAG antibody was purchased from Sigma (anti-FLAG-M2 monoclonal). Anti-FLAG antibody was purchased from Sigma (anti-FLAG-M2 monoclonal antibody), anti-mt-HSP70 was from Affinity Bioreagents, and anti-calnexin and anti-cytochrome c antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Retroviral Infections—Retroviruses were produced by transfecting the Phoenix helper cell line with 5–10 μg of pBABE-based constructs. 48 h after transfection, supernatants were collected and passed through a 0.2-μm filter. After the addition of 10 μl of a 10 mg/ml stock solution of polybrene, the supernatants were added to MEFs plated on 10-cm dishes. Supernatants were removed approximately 3 h after infection, and a second cycle of infection was repeated. 48 h after infections,
selection was performed by adding puromycin to a concentration of 2 \(\mu g/mL\). Selection was maintained for 4 days, and surviving cells were used for further experiments.

**Plasmids and Transfections—Mito-GFP** was a gift from Dr. Rizzuto (University of Padova). The plasmid encoding matrix protein peptidase \(\alpha\) was a gift from Dr. Rapaport (University of Munich). The coding sequences for p46, p52, and p66Shc had been previously isolated in our laboratory. All experiments described in this paper were performed using Shc constructs of human origin unless otherwise stated. Shc cDNAs were subcloned in pBABE vectors to obtain the retroviral constructs and subcloned in pCDNA3.1 for transient transfection experiments. Since p52 and p46Shc are translated from the same mRNA, via alternative ATG usage, the p46 ATG was mutated to TTG in the p52Shc expressing constructs (3).

To generate the N-terminally tagged FLAG constructs, the coding sequence of p46Shc, p52Shc, and p66Shc as well as the regions corresponding to the PTB of p46Shc and of p52Shc and the CH1–SH2 region were amplified by PCR using oligonucleotides designed to generate an EcoRI restriction site at the 5’- and 3’-end (excluding the STOP codon) and cloned in frame with the FLAG sequence in the pCMV-Tag4 vector. The N-terminally tagged p46Shc was generated by cloning the p46Shc coding sequence into the EcoRI site of pCDNA3.1 FLAG A, immediately downstream of the FLAG sequence.

p46RFP was generated by cloning the p46 EcoRI inserts from pCMV-TAG4 constructs into EcoRI-digested pDSRed-N1 vector (Clontech), in frame with the coding sequence of the red fluorescent protein. To generate the (1-52)p46RFP construct, the sequence coding for the first 32 amino acids of p46Shc was amplified by PCR and cloned in pDSRed-N1.

To generate deconstruct constructs, 5’-oligonucleotides with a HindIII site were used together with a common 3’-oligonucleotide to amplify the full-length cDNAs. To reduce the risk of introducing unwanted mutations, PCR products were digested with HindIII and BamHI (which cuts in the region coding for the PTB), and the resulting fragments were cassetted into pCDNA3.1-p52Shc-digested HindIII-BamHI. One exception is the p46Shc11 construct, which we had previously isolated as a naturally occurring splice variant originating from the splicing of exon 1 directly to exon 3. For all constructs, the entire region generated by PCR was sequenced to assure fidelity. The oligonucleotides used are shown in Table I.

**Mitochondrial Localization of p46Shc**

- **Table I**

| Oligonucleotide | Sequence |
|----------------|----------|
| p52A10        | 5’-CGGAAGCTTGACATGACAGGACTCGGGTGAGGAGGGGCG-3’ |
| p52A30        | 5’-CGGAAGCTTGACATGACAGGACTCGGGTGAGGAGGGGCG-3’ |
| p52A40        | 5’-CGGAAGCTTGACATGACAGGACTCGGGTGAGGAGGGGCG-3’ |
| p46A32        | 5’-CGGAAGCTTGTCATGGGAGCCCTGAGTTCAACCCCGGG-3’ |
| p46A32        | 5’-CGGAAGCTTGTCATGGGAGCCCTGAGTTCAACCCCGGG-3’ |
| “Common” AS   | 5’-CGGAAGCTTGTCATGGGAGCCCTGAGTTCAACCCCGGG-3’ |

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were sequenced for the presence of the desired mutation and the absence of other, unwanted, base changes. For the generation of p46Shc point mutants, oligonucleotides listed in Table II were synthesized. Immunochemistry—Cells plated on glass coverslips that had been preincubated with 0.1% gelatin in PBS at 37 °C for 30 min were fixed in 4% paraformaldehyde in PBS for 10 min, washed with PBS, and permeabilized by incubating in PBS, 1% Triton X-100 for 10 min at room temperature. To prevent nonspecific binding of the antibodies, cells were then incubated with PBS in the presence of 5% bovine serum albumin for 30 min. The coverslips were then gently deposited, face down, on 100 μl of primary antibody diluted in PBS, 5% bovine serum albumin. After 1 h, coverslips were washed three times with PBS (5 min per wash). Cells were then incubated for 30 min at room temperature with the appropriate secondary antibody Cy3 (Amersham Biosciences), Alexa 488- or Alexa 350-conjugated (Molecular Probes, Inc., Eugene, OR). Coverslips were mounted in a 90% glycerol solution containing diazabicyclo(2.2.2)octane antifade (Sigma) and examined under an AX-70 Provis (Olympus) fluorescence microscope equipped with a black and white cooled CCD camera (Hamamatsu c9885) and with a Bio-Rad MRC1024 confocal microscope equipped with a 20-milliwatt krypton-argon laser for colocalization analysis. Confocal image acquisition was performed in sequential mode to limit channel cross-talk and corrected for residual fluorescence breakthrough. Green fluorescence was collected through a 520 ± 35-nm band pass filter, whereas the red channel filter was a 605 ± 20-nm band pass. Images were further processed with the Adobe Photoshop software (Adobe).

**Mitochondrial Staining with Mitotracker Red**—Cells growing on coverslips were incubated for 15 min with Mitotracker Red (Molecular Probes) diluted 1:2000 in complete cell culture medium, washed with PBS, and then fixed with 4% paraformaldehyde in PBS.

**Isolation and Subfractionation of Mitochondria**—Cells were washed with PBS and collected by scraping in the same buffer. Cellular pellet was resuspended, adding 5 volumes of MT buffer (Tris/MOPS (10 mM), pH 7.4, 1 mM EGTA, 250 mM sucrose), and subjected to 20 hand strokes of a Dounce homogenizer (at visual inspection, at least 90% of the cells were broken). Nuclei and unbroken cells were pelleted twice for 5 min at 800 × g. Mitochondria were finally isolated at 8000 × g and washed once in MT buffer.

To identify the submitochondrial localization of the proteins of interest, equal aliquots of organelles (50–100 μg in 100 μl) were left untreated or subjected to proteinase K treatment (50 μg/ml for 20 min on ice). The protease digestion was conducted either in MT buffer, digesting proteins resident at the external surface of the outer membrane, or into hypotonic buffer (MT without sucrose), leading to outer membrane removal and digestion of intermediate space proteins, leaving intact the mitoplast. The protease was stopped, adding the inhibitor phenylmethylsulfonyl fluoride at a 2 mM concentration, followed by a 5-min incubation on ice. Organelles were resolubilated at 15,000 × g for 10 min and washed twice in MT, 1 mM phenethylmethylsulfonyl fluoride. Mitoplast preparations were analyzed as such or further subfractionated, treating them with 50 μl of 0.1 M sodium carbonate, pH 11.5, and left for 30 min on ice. Unbroken material was pelleted for 10 min at 15,000 × g, and the supernatant was centrifuged for 30 min at 100,000 × g. Proteins present in the pellet (membrane fraction containing integral membrane proteins) and supernatant (matrix and extractable membrane proteins) were resolved by SDS-PAGE and analyzed by Western blotting.

**Mitochondrial Import Assay**—Proteins were expressed as \(^{35}S\) methionine-labeled, using a commercially available transcription/translation reticulocyte system (Promega). 50 μg of mitochondria, prepared from CV-1 cell as described, were incubated in a final volume of 200 μl of MT buffer (20 mM Hepes-KOH, pH 7.2, 250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 4% bovine serum albumin, 2 mM ATP, 5 mM NAD, 0.5 mM thiotimidine). After transcription/translation, the reticulocyte mixture was centrifuged at 100,000 × g for 1 h, and 10 μl of supernatant was added to the mitochondria. The import incubation was 20 min at 30 °C under mild agitation. The incubated mixture was divided into two halves. One half was mock-treated, and the other was proteinase K-treated (conditions of treatment, stopping, and wash-
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**RESULTS**

**P46Shc Is a Mitochondrial Protein**—p46Shc is entirely contained within the p66Shc and p52Shc sequence, thereby precluding the generation of p46Shc-specific antibodies. To overcome this limitation and to avoid potential artifacts connected with the utilization of epitope-tagged cDNAs, we used recombinant retroviruses encoding for single Shc isoforms to reconstitute p46, p52, and p66Shc expression into mouse embryo fibroblasts from Shc null embryos (Shc−/− MEFs) (11) (Fig. 1, A and B). After selection with puromycin, the infected Shc−/− MEFs were analyzed by indirect immunofluorescence, using an anti-pan-Shc antibody (anti-Shc-SH2). p52 and p66Shc staining was mainly cytoplasmic diffuse, whereas p46Shc localized almost exclusively to discrete cytosolic structures whose shape and distribution was reminiscent of mitochondria (Fig. 1C).

We next performed co-localization experiments using the mitochondria-specific dye MitoTracker Red. p46Shc co-localized with mitochondria, whereas the staining patterns of p52 and p66Shc only partially overlapped with that of these organelles (Fig. 1D). Identical results were obtained when the mitochondria were visualized by expressing a green fluorescent protein targeted to mitochondria (MitoGFP) or with an anti-cytochrome c antibody (not shown).

To investigate whether a fraction of endogenously expressed Shc proteins localizes to mitochondria, we co-stained NIH3T3 cells, which express p46Shc together with p52Shc and p66Shc, with Mitotracker and the anti-pan-Shc antibody. A partial, yet unambiguous, overlap was evident (Fig. 2A).

Four distinct compartments can be distinguished within mitochondria: the outer membrane, the inter membrane space, the inner membrane, and the matrix. In order to investigate the subcellular compartmentalization of Shc proteins, we carried out subcellular and submitochondrial fractionation experiments. Purified mitochondria were treated with proteinase K (PK) to remove all nonmitochondrial proteins and proteins associated with the outer mitochondrial membrane (Fig. 2B). Similarly to cytochrome c (an intermembrane space protein) and to mtHsp70 (a matrix protein), p46Shc was almost completely resistant to PK treatment, thus indicating that either it is an integral inner membrane protein or it resides in the intermembrane space or in the mitochondrial matrix. To discriminate between these possibilities, purified mitochondria were resuspected in a hypotonic buffer, which induces breakage of the outer membrane, and then treated with PK (Fig. 2B; mitoplasts). Significant PK digestion of cytochrome c, but not of mtHsp70, was observed, as expected. Again, the majority of p46Shc was resistant to PK digestion (Fig. 2B). A smaller fraction

**TABLE II**

| Oligonucleotide | Sequence |
|-----------------|----------|
| R175Q S         | 5′-CAAGACCCCGTGAATCCCGAGGCCTGCCACATTCTGGAGCTGCC-3′ |
| R175Q AS        | 5′-GAGACCCCGTGAATCCCGAGGCCTGCCACATTCTGGAGCTGCC-3′ |
| R112Q/K116A S   | 5′-CAGCTCTATCTCGGCGAGTACCTGCGCTATTCTGGAGCTGCC-3′ |
| R112Q/K116A AS  | 5′-GAGCTATCGCAGATTCATATGAGCGTACCTGCGCTATTCTGGAGCTGCC-3′ |
| R139A S         | 5′-CTGCCGACCCGCTGCCACATTCTGGAGCTGCC-3′ |
| R139A AS        | 5′-GAGCTATCGCAGATTCATATGAGCGTACCTGCGCTATTCTGGAGCTGCC-3′ |
| R10E S          | 5′-GCCTCAAAACCATGTACTCAGGACCTGCGCTATTCTGGAGCTGCC-3′ |
| R10E AS         | 5′-GGTTTCTCTGTTGGTTGAGTGATACTGTTGTTGAGCTGCC-3′ |
| R22E S          | 5′-GGTTTCTCTGTTGGTTGAGTGATACTGTTGTTGAGCTGCC-3′ |
| R22E AS         | 5′-GGTTTCTCTGTTGGTTGAGTGATACTGTTGTTGAGCTGCC-3′ |

**FIG. 1.** Subcellular localization of Shc isoforms. A, schematic representation of the cDNAs used for the expression of the three Shc isoforms. Notice that to obtain the expression of only p66Shc, the start codons for p46Shc and p52Shc were mutated to TTG (red crosses). Similarly, in the cDNA coding for p52Shc only, the p46Shc start codon was changed to TTG. B, lysates from Shc−/− MEFs were with pBABE (lane 1) or with retroviruses expressing the cDNA for p46Shc (lane 2), p52Shc (lane 3), or p66Shc (lane 4) were subjected to Western blotting using an anti-Shc-SH2 antibody. A lysate from uninfected wild type MEFs (lane 5) was used as a positive control. C, immunofluorescence analysis of Shc−/− MEFs expressing the indicated Shc isoforms and decorated with an anti-Shc-SH2 antibody. D, Shc−/− MEFs expressing the indicated Shc isoforms were incubated with the mitochondrial dye Mitotracker Red, decorated with an anti-Shc-SH2 antibody, and analyzed by confocal microscopy.

of p52 and p66Shc also co-fractionated with mitochondria, whose specificity and significance is currently under investigation.

To further discriminate whether p46Shc resides within the...
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inner membrane or the matrix, mitoplasts were incubated with 0.1 M sodium carbonate (pH 11.5), which induces the release of soluble and peripheral membrane proteins (12) (Fig. 2C). As expected, the majority of the matrix protein mtHsp70 was found in the supernatant, whereas the integral inner membrane protein cytochrome oxidase IV (COXIV) remained associated with the membrane fraction (Fig. 2C). Notably, p46Shc was found in the supernatant, indicating that this Shc isoform resides in the mitochondrial matrix either as a soluble protein or peripherally associated with the inner face of the inner mitochondrial membrane (Fig. 2C).

The PTB of p46Shc Is Necessary and Sufficient for Mitochondrial Targeting—The unexpected subcellular localization of p46Shc raises a number of questions regarding the molecular nature of the targeting mechanism at work. As shown in Fig. 1A, the only difference between p46Shc and p52Shc resides in a 45-amino acid amino-terminal extension present in p52Shc. The reported three-dimensional structure of the p52Shc PTB, determined by nuclear magnetic resonance (13), indicates that its first 40 amino acids assume a disordered configuration and do not take part directly to the folding of the domain. This region could affect the mitochondrial targeting of p52Shc in at least two ways. First, an N-terminal mitochondrial targeting sequence on p46Shc could be “masked” in p52Shc. Second, if the mitochondrial localization of p46Shc is mediated by the binding of the PTB to some mitochondrial phosphoproteins or phospholipids, the presence of the N-terminal extension in p52Shc could affect specificity and/or affinity of this interaction. In order to discriminate between these two possibilities, we examined whether the PTB region of p46Shc is necessary and sufficient for mitochondrial targeting and, if so, whether binding to phosphorytrosine-containing proteins or phospholipids is required.

To evaluate the role of the PTB in mitochondrial targeting of p46Shc, we prepared constructs expressing the PTB of p46Shc, the PTB of p52Shc, or the CH1-SH2 portion common to both isoforms (ΔPTB-Shc). To allow easy tracking of their subcellular localization, a C-terminal FLAG epitope was appended to each construct. NIH3T3 cells were transiently transfected with these plasmids, stained with Mitotracker Red, and subjected to indirect immunofluorescence with an anti-FLAG monoclonal antibody. The PTB of p46Shc, but not that of p52Shc or the CH1-SH2 portion, was efficiently targeted to mitochondria (Fig. 3A). The partial nuclear localization observed with the p52PTB is probably due to free diffusion of this small molecule (about 20 kDa). These results demonstrated that the PTB domain of p46Shc is necessary and sufficient for mitochondrial targeting.

The PTB domain of Shc has been reported to bind phosphorytrosine-containing proteins and a subset of phospholipids (13), the latter being a property that stems from the close similarity between the structure of PTB and pleckstrin homology domains. The mitochondrial targeting of the p46Shc PTB could thus be due to the binding property of the PTB or, alternatively, to a specific sequence within the Shc PTB. Point mutations that abolish either phosphorytrosine or phospholipid binding have been described (14). In particular, a single mutation in the PTB (R175Q; the numbering refers to p52Shc) abolishes binding to phosphorytrosines, whereas a triple mutation (R112Q/K116A/K139A) interferes with the binding to phospholipids. We introduced these mutations into the p46Shc cDNA and tested whether they affected the subcellular localization of p46Shc. As shown in Fig. 3B, both p46ShcR175Q and p46ShcR112Q/K116A/K139A were efficiently targeted to mitochondria. Thus, although the PTB region is required for mitochondrial targeting of p46Shc, it does not mediate this targeting through its ability to bind phosphorylated proteins or phospholipids.

The p46Shc N Terminus Is a Bona Fide Mitochondrial Targeting Sequence—Our results indicate that the PTB domain of p46Shc contains a signal that is necessary and sufficient for mitochondrial targeting. To map its location, we generated a series of amino-terminal deletion mutants of p46Shc (Fig. 4A) and examined their subcellular localization (Fig. 4B). Deletion of the first 11 amino acids of p46 (as in the p46Δ11 mutant) did not affect p46Shc mitochondrial targeting, whereas a larger deletion, encompassing amino acids 1–22 or 1–32, completely disrupted targeting to this organelle (Fig. 4B; p46Δ32 and p46Δ32 mutants). Together, these results indicate that an important determinant for mitochondrial targeting resides between amino acids 12 and 22 of p46Shc.
Most nucleus-encoded mitochondrial proteins are directed to mitochondria via an amino-terminal targeting sequence (15). One important feature of these signals is that they act independently of the nature of the remaining of the protein. To assess whether the p46Shc mitochondrial targeting sequence shares this characteristic, we prepared constructs in which the entire p46Shc coding sequence or only its first 32 amino acids were fused, in frame, to the cDNA of the red fluorescent protein (RFP), a protein unrelated to p46Shc that emits a bright red fluorescence (16). The resulting constructs, named RFP, p46RFP, and (1–32)p46RFP, were transfected with the plasmids encoding for the indicated p46Shc mutants were stained with Mitotracker Red, decorated with an anti-Shc-SH2 antibody, and analyzed by immunofluorescence microscopy. As previously reported (16), the RFP alone displayed a nuclear and cytoplasmic diffuse staining, whereas the p46RFP fusion protein mirrored the behavior of p46Shc, strongly co-localizing with the MitoGFP (Fig. 5, RFP and p46RFP). More importantly, the presence of the first 32 amino acids of p46Shc at the amino terminus of the RFP was sufficient to induce mitochondrial localization of the corresponding chimeric protein (Fig. 5, (1–32)p46RFP).

The p46Shc N terminus is not recognized as a potential mitochondrial targeting signal by currently available software (MitoProt II, TargetP; available on the World Wide Web at www.cbs.dtu.dk/services/TargetP/ and (Predotar) www.inra.fr/predotar/). However, visual inspection of the sequence revealed the presence of three arginine residues in the first 29 amino acids (positions 10, 22, and 29) as well as several hydrophobic and hydroxylated amino acids (Fig. 6A). Since mutation of a single arginine residue (Arg23) in the targeting sequence of ornithine transcarbamylase is sufficient to completely abrogate mitochondrial import (17), we examined the subcellular localization of the first 32 amino acids of p46Shc and the RFP ((1–32)p46RFP). Cells were examined by epifluorescence microscopy 36 h after transfection.
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Fig. 6. Efficient mitochondrial targeting of p46Shc critically depends on an arginine residue at position 22. A, amino acid sequence of the p46Shc N terminus. The mutagenized arginine residues are represented in red. B, NIH3T3 cells were transfected with the wild type or mutant p46ShcFLAG constructs, as indicated, and then, after 36 h, incubated in the presence of Mitotracker Red, fixed, decorated with an anti-FLAG antibody, and examined by immunofluorescence microscopy. C, lysates of NIH3T3 cells transfected with the indicated constructs were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and probed with an anti-FLAG antibody. D, in vitro import experiment. The matrix protein peptidase α (MPP), p46FLAG, and p46FLAG R22E were in vitro-translated in presence of [35S]methionine and incubated with mitochondria purified from CV-1 cells, either in the presence or absence of valinomycin. After washing, nonimported proteins were digested by the addition of proteinase K (third and fourth lanes from left).

ished mitochondrial targeting, whereas the mutation of arginine 10 or of arginine 29 to glutamate had no significant effect on the subcellular localization of p46Shc (Fig. 6B).

Import into the mitochondrial matrix requires the presence of a membrane potential across the inner membrane (Δψ) (16). To test whether p46Shc had a similar requirement, we performed in vitro import experiments using mitochondria purified from CV-1 cells. Although less efficiently than the matrix protein peptidase α, p46ShcFLAG was imported into purified mitochondria in a Δψ-dependent manner, as demonstrated by the sensitivity to valinomycin (Fig. 6D). Consistently with the immunofluorescence experiments, no import was observed with the p46Shc R22E mutant. Another common feature of most proteins residing in the mitochondrial matrix is that their N-terminal signal is cleaved upon import by a specific mitochondrial matrix peptidase. This does not seem to be the case with p46Shc, since the electrophoretic mobility of wild type p46Shc was indistinguishable from that of the R22E mutant (Fig. 6, C and D). We note, however, that due to the limited resolution of SDS-PAGE, the possibility that a very small signal peptide is removed from p46Shc after import cannot be formally excluded. All together, these results indicate that p46Shc is targeted to the mitochondria via an N-terminal signal peptide that behaves as a bona fide mitochondrial targeting sequence.

The Function of the p46Shc Mitochondrial Targeting Signal Is Inhibited in the Context of p52Shc and p66Shc —The unique ability of p46Shc, as compared with the other Shc isoforms, to localize to mitochondria raises a number of questions regarding the underlying targeting mechanism. All three isoforms, indeed, contain the mitochondrial localization signal identified in p46Shc and differ by the presence of overlapping amino-terminal regions of different lengths. It is plausible that the inability of the PTB to efficiently target p52 and p66Shc to the mitochondria is due to its being masked by the N-terminal extensions present in these two isoforms. To investigate this possibility, we prepared a series of amino-terminal deletion mutants of p52Shc and examined their localization by indirect immunofluorescence (Fig. 7, A and B). Notably, all the mutants that retained a portion of the p52Shc-specific N-terminal extension (even if only 5 amino acids, as in the p52Δ40 mutant) were found in the cytosol (Fig. 7B). To test whether a free N terminus is an absolute requirement for the mitochondrial localization of p46Shc, we prepared two p46Shc constructs, differing exclusively by the presence of a FLAG epitope appended to the N terminus (FLAGp46) or the C terminus (p46FLAG) of the p46Shc cDNA (Fig. 8, A and B). Strikingly, whereas p46FLAG retained the mitochondrial localization, the FLAGp46 fusion protein showed a diffuse cytoplasmic staining indistinguishable from that of p52FLAG and p66FLAG (Fig. 8C). Together,
the different subcellular localization of p46Shc and p52/p66Shc.
requirement for mitochondrial targeting of p46Shc and explain these results demonstrate that a free N terminus is an absolute

(19). Other examples include the phosphorylation on tyrosine
responsible for the interaction of p52 (and p66Shc) with c-Src residues 29 and 45 of p52Shc (which is absent in p46Shc) is times lower for p46Shc (18), and the region comprised between
for phosphotyrosine-containing proteins, which is about 10
N-terminal extension in p52Shc affects the affinity of the PTB
been previously reported. For example, the presence of the
dence of a functional divergence between p46- and p52Shc had
identical to p52Shc. Its localization to mitochondria, however,
signal, although present also in p52Shc and p66Shc, is non-
localize in mitochondria or at the mitochondrial surface. Par-
deed, other signal transducer proteins have been reported to
phosphotyrosine-based signaling pathway in mitochondria. In-

Fig. 8. The mitochondrial targeting signal of p46Shc has to be positioned at the N terminus to be functional. A, schematic rep-
resentation of the epitope-tagged Shc isoforms used in the experiments reported in B and C. The position of the FLAG epitope is indicated by a red box with a green flag. B, lysates from NIH3T3 cells transfected with the indicated plasmids were resolved by SDS-PAGE and probed with an anti-FLAG antibody. C, immunofluorescence analysis of NIH3T3 cells transfected with the indicated plasmids, stained with Mitotracker and decorated with the anti-FLAG antibody.

these results demonstrate that a free N terminus is an absolute requirement for mitochondrial targeting of p46Shc and explain the different subcellular localization of p46Shc and p52/p66Shc.

DISCUSSION

In this study, we demonstrate that a significant fraction of p46Shc resides in the mitochondrial matrix, as assessed by both immunofluorescence microscopy and subcellular fractionation studies. Using a combination of site-directed mutagenesis, deletion mapping, and chimeric fusion proteins, we identify a noncleaved mitochondrial targeting signal at the N terminus of p46Shc, whose activity is strongly dependent on the presence an arginine residue at position 22. We further show that this signal, although present also in p52Shc and p66Shc, is non-functional in these two Shc isoforms due to its internal position.

p46Shc has been generally considered to be functionally identical to p52Shc. Its localization to mitochondria, however, strongly suggests that this may not be the case. Indeed, evidence of a functional divergence between p46- and p52Shc had been previously reported. For example, the presence of the N-terminal extension in p52Shc affects the affinity of the PTB for phosphotyrosine-containing proteins, which is about 10 times lower for p46Shc (18), and the region comprised between residues 29 and 45 of p52Shc (which is absent in p46Shc) is responsible for the interaction of p52 (and p66Shc) with c-Src (19). Other examples include the phosphorylation on tyrosine residues of p52Shc, but not of p46Shc, in response to insulin treatment (20), the phosphotyrosine-independent interaction between the PTB of p52Shc and p66Shc and the protein phosphatase PEST (21), and the recent demonstration, by Faisal et al. (22), that phosphorylation of Ser^29 of p52Shc (Ser^138 in p66Shc) after treatment with phorbol 12-myristate 13-acetate or insulin (but not with epidermal growth factor nor fibroblast growth factor-2) is required for the isofrom-specific binding to protein phosphatase PEST.

A number of important similarities between p46Shc and p52Shc also have to be considered. For example, epidermal growth factor treatment induces tyrosine phosphorylation of p46Shc and p52Shc, and both isoforms bind the activated receptor and Grb2. How can these data be reconciled with the mitochondrial localization of p46Shc? That a significant fraction of p46Shc resides in the cytosol is confirmed by our subcellular fractionation experiments showing that 10–30% of p46Shc remains in the mitochondria-free supernatant after differential centrifugation (Fig. 2 and data not shown). Although p46Shc is strongly enriched in the mitochondrial fraction of all the cell types and tissues tested so far, we have observed a significant variability in the amount of extramitochondrial p46Shc. In this regard, it will be important to understand how the ratio between cytosolic and mitochondrial p46Shc is determined and maintained. One possibility is that p46Shc continuously shuttles between the mitochondria and the cytosol and that a fraction of it is available for binding to activated receptor-tyrosine kinases. Although formally possible, this is an unlikely scenario, since to our knowledge there are no known examples of proteins shuttling between the mitochondrial matrix and the cytosol. Alternatively, two independent pools of p46Shc could coexist in the cell, one in the mitochondrial matrix, serving an as yet unidentified function, and another in the cytoplasm, ready to be recruited to activated receptor-tyrosine kinases or to serve as a substrate for other tyrosine kinases. In this scenario, the sorting of newly synthesized p46Shc molecules to one compartment or the other could be a stochastic event or could be regulated by posttranslational modifications, as was previously described for CYP2E1 (23) or by the availability of a binding partner for the p46Shc PTB. In this regard, it has been recently shown that the conformation of the PTB of Shc is dramatically affected by binding to a phosphorylated TrkA peptide (24).

Mitochondria play a central role in apoptosis and in the regulation of cellular metabolism, two functions that must be tightly controlled in response to many different intracellular and extracellular stimuli. To our knowledge, p46Shc represents the first example of a PTB- and SH2-containing protein that localizes to the mitochondrial matrix. The fact that Shc is an adapter protein, devoid of enzymatic activity, and is the substrate of many tyrosine kinases, suggests the existence of a phosphotyrosine-based signaling pathway in mitochondria. Indeed, other signal transducer proteins have been reported to localize in mitochondria or at the mitochondrial surface. Particularly interesting is A-Raf, a member of the Raf family of serine protein kinases, which has been shown to reside in the mitochondrial matrix (25) and whose function in this organelle is still unknown. Other protein kinases localizing to mitochondria include Raf1, which is recruited to the outer mitochondrial membrane by the anti-apoptotic protein Bcl2 (26). Jun N-terminal kinases have also been reported to translocate to mitochondria in response to DNA damage (27) and to treatment with phorbol 12-myristate 13-acetate (28). Other examples include protein kinase C6, which has been shown to alter the mitochondrial membrane potential and induce apoptosis in response to phorbol 12-myristate 13-acetate treatment (29) and
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oxidative stress (30), and the c-Abl tyrosine kinase, which also is recruited to mitochondria in response to oxidative stress (31). Finally, other as yet unidentified protein kinases are likely to exist in mitochondria; tyrosine kinase activity in mitochondria has in fact been detected in mitochondria from human placenta (32), and a cAMP-independent kinase activity phosphorylates proteins of 44-, 39-, and 31 kDa in bovine heart mitochondria (33).

Based on these findings, it is tempting to speculate a possible role of p46Shc in signal transduction pathways affecting mitochondrial physiology. To date, however, we failed to detect phosphorylation on tyrosine residues of the mitochondrial fraction of p46Shc in response to mitogenic (platelet-derived growth factor and epidermal growth factor) or apoptotic (staurosporine, UV irradiation, and oxidative stress) stimuli (data not shown). Similarly, these stimuli did not significantly affect the ratio of mitochondrial to cytosolic p46Shc (data not shown).

Another finding presented here is the identification of a cryptic mitochondrial targeting signal that allows the specific localization of p46Shc, but not of p52/p66Shc, to mitochondria. A similar mechanism has been previously described for D-AKAP1 (34), a protein belonging to the family of the A kinase-anchoring proteins, whose function is to target the cAMP-dependent protein kinase to specific subcellular compartments. The D-AKAP1 gene encodes for two amino-terminal splice variants (N0 and N1), with N1 having a 33-amino acid extension respect to N0. Analogously to what we report for p46Shc, the shorter isoform localizes to mitochondria, whereas the longer one is found in the endoplasmic reticulum (34).

The results reported in this study, together with previously published data (2, 8, 9), demonstrate a high degree of functional specialization and divergence among the various Shc isoforms and suggest that p46Shc might exert important functions in signal transduction pathways regulating mitochondrial physiology. Further studies aimed at the identification of p46Shc-interacting proteins in the mitochondria and examining the consequences of p46Shc loss on mitochondrial function are warranted and will probably shed light on the biology of this interesting adapter molecule.

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