Localization of Endogenous Grb10 to the Mitochondria and Its Interaction with the Mitochondrial-associated Raf-1 Pool*

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Grb10 belongs to a small family of adapter proteins that are known to interact with a number of receptor tyrosine kinases and signaling molecules. We have recently demonstrated that the Grb10 SH2 domain interacts with both the Raf-1 and MEK1 kinases. Overexpression of Grb10 genes with mutations in their SH2 domains promotes apoptosis in cultured cells, a phenotype that is reversed by concomitant overexpression of the wild type gene. Using immunofluorescence microscopy and subcellular fractionation we now show that most of the Grb10 molecules are peripherally associated with mitochondria. Following insulin-like growth factor 1 or serum treatment, small pools of Grb10 can also be found at the plasma membrane and in actin-rich membrane ruffles, whereas overexpression of Grb10 leads to its mislocalization to the cytosol. Two-hybrid analysis shows that the Grb10-binding site on Raf-1 co-localizes with its Ras-binding domain. Finally, we show that the endogenous Grb10 and Raf-1 proteins can be co-immunoprecipitated from a partially purified mitochondrial extract, an interaction that is enhanced following the activation of Raf-1 by ultraviolet radiation. Thus, we infer that Grb10 may regulate signaling between plasma membrane receptors and the apoptosis-inducing machinery on the mitochondrial outer membrane by modulating the anti-apoptotic activity of mitochondrial Raf-1.

Grb10 (1) and its close homologues Grb7 (2) and Grb14 (3) are considered to be adapter proteins because they interact with several signaling proteins and lack any apparent enzymatic activity (see Ref. 4 for review). Collectively, they are termed the Grb7 family and share a carboxyl-terminal SH2 domain that binds to several activated receptor tyrosine kinases, a central Pleckstrin homology (PH) domain, and a small proline-rich sequence that can interact with SH3 domains in vitro (5). The BPS domain, located between the PH and SH2 domains of Grb10 and Grb14, interacts with activated insulin-like growth factor 1 (IGF-I) and/or insulin receptors (6, 7). Finally, sequence analysis suggests the existence of a Ras-associating-like domain (8).

Several tumors and cell lines show increased expression of various members of the Grb7 family. In addition, epidemiological and experimental evidence links Grb7 overexpression to extramucosal tumor invasion in human esophageal carcinoma (9, 10). A specific role for Grb10 is less clear. It interacts with the insulin, IGF-I, epidermal growth factor, Eph family receptor, and growth hormone receptors, the Ret protooncogene, and other signaling molecules such as Jak2, Bcr-Abl, and Tec (11, 11–14). We have previously demonstrated that the Grb10 SH2 domain interacts with the Raf-1 and MEK1 kinases (15); both are components of the mitogenic MAP kinase signal transduction pathway that transmits hormonal signals to proliferative or differentiation events (see Refs. 16 and 17 for reviews). Stably transfected cell lines overexpressing Grb10 display moderate inhibition of cell cycle progression and alterations in other aspects of their signaling pathways (11, 14, 18). Finally, overexpression of full-length hGrb10 genes carrying SH2 domain mutations promotes apoptosis in HTC-IR and COS-7 cells. This cell death phenotype can be reversed by concomitant overexpression of the wild type hGrb10 gene (15).

The serine/threonine kinase Raf-1 transmits mitogenic signals as part of the Raf-MEK-ERK kinase cascade, although only a small proportion of total Raf-1 molecules are translocated to the plasma membrane by Ras and activated by other proteins (19, 20). Recent work has revealed that Raf-1 is implicated in many other signaling events. During mitosis, cytoplasmic Raf-1 is activated independently of Ras, and its function does not include activation of the downstream MEK and ERK kinases (21). Furthermore, depending on the cell line or its intracellular localization, Raf-1 can act either as a promoter or as an inhibitor of apoptosis (22–25). For example, Raf-1 can be targeted to mitochondria by Bcl-2, resulting in increased resistance to apoptotic inducers (26, 27). Such variation in function requires a complex regulatory system; Raf-1 can interact with a large number of proteins including Ras, 14-3-3, Hsp90, Csk, Ksr, MEK1, and others (20, 28–33).

To determine how Grb10 affects the activity or intracellular localization of its ligands, we attempted to confirm the intracellular localization of the endogenous protein by immunofluorescence microscopy and cell fractionation. Previous results suggested that Grb10 is a soluble protein that becomes localized to the plasma membrane following insulin treatment (5, 34). However, these investigations used antibodies that recognize several bands on immunoblots (possibly because they were

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1 The abbreviations used are: PH, Pleckstrin homology; CoxI, cytochrome oxidase I; FBS, fetal bovine serum; IGF-I, insulin-like growth factor I; mAb, monoclonal antibody; MP, medium speed pellet; SH2, Src homology 2; MAP, mitogen-activated protein; ERK, extracellular-regulated kinase; MEK, mitogen-activated ERK kinase; PBS, phosphate-buffered saline.

2 We use a unified nomenclature for the naming of Grb10 splice variants that has been agreed upon by several researchers and is maintained on the World Wide Web (15).
Mitochondrial Localization of Grb10

Production of anti-Grb10 Antibodies—We subcloned a FLAG-tagged hGrb10(R520L) gene (15) in the EcoRI/XhoI sites of pFastBac1 and produced recombinant baculoviruses with the Bac-to-Bac Baculovirus Expression System (Life Technologies, Inc.). Expression of the wild type hGrb10 protein was found to inhibit large scale viral multiplication, whereas the baculovirus protein p35 possibly protected the hGrb10(R520L)-expressing SF9 cells from apoptosis (38). The expressed protein was purified by affinity chromatography on an anti-FLAG(M2) resin as described (39). Antibodies were raised by inoculating rabbits with 100 μg of hGrb10(R520L) in Titer Max adjuvant (CytRx Corporation). Anti-Grb10 antibodies were affinity-purified with maltose-binding protein-hGrb10-coupled activated CH Sepharose 4B (Amersham Pharmacia Biotech).

Cell Culture and Immunofluorescence Microscopy—COS-1 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium + 10% FBS. To prepare the cells for immunofluorescence microscopy, we inoculated 5,000–15,000 cells into the wells of an 8-chambered glass or Permanox slide (Nunc). The next day, the cells were fixed 10 min in 4% paraformaldehyde, permeabilized for 2 min in 0.2% Triton X-100 in PBS, and maintained in serum-free media for 4 h.

For immunofluorescence microscopy, we inoculated 5,000–15,000 cells into the wells of a 6-chambered glass or Permanox slide (Nunc). The next day, the cells were fixed 10 min in 4% paraformaldehyde, washed twice in PBS, permeabilized for 2 min in 0.2% Triton X-100 in PBS, and washed twice more in PBS. They were then blocked for 30 min in PBS + 10% FBS and incubated for 60 min with the primary antibodies in PBS + 10% FBS. The samples were washed four times with PBS, incubated 30 min with the fluorescent secondary antibodies, and washed four more times in PBS. Slides were mounted in ProLong antifade reagent (Molecular Probes) and viewed with a Leitz Aristoplan microscope coupled to a Princeton Instrument CCD camera. Images were subsequently analyzed with Eclipse (Empix Imaging Inc.) and Photoshop (Adobe) software. Localization of Grb10 to the mitochondria and membrane ruffles were also observed with a Nikon Diaphot confocal microscope. The concentration of primary antibodies used were 1:100 for the preimmune and anti-Grb10 serum, 0.3 μg/ml for affinity-purified anti-Grb10 and 1:50 for the anti-CoxII antibodies (Molecular Probes). Specific fluorochrome isothiocyanate, lissamine rhodamine, and Texas Red-coupled secondary antibodies were from Jackson ImmunoResearch. Fluorescent Rhodamine was obtained from Sigma and used at a dilution of 1:50. Although the quality and intensity of the signals were reduced, similar conclusions were reached with variations of the original protocol such as methanol fixation or using bovine serum albumin as a blocking agent.

Cell Fractionation—HeLa S3 cells were spun down, washed twice in PBS, and resuspended in 1 ml of extract with either 500 ng of Santa Cruz Biotechnology Raf-1 (E10) mAb or 1 μg of either the preimmune or anti-Grb10 sera. After overnight incubation at 4 °C, the antibodies were precipitated with protein A-Sepharose (Amersham Pharmacia Biotech), washed extensively in MS buffer, and boiled in SDS-polyacrylamide gel electrophoresis buffer. Raf-1 activity in immunoprecipitates was determined as described (42) except that the amount of activated ERK1 was estimated with a phospho-specific antibody (New England Biolabs).

Production and Characterization of Grb10 Antibodies—The hGrb10(R520L)-FLAG protein (see “Materials and Methods”) was expressed in insect cells with a baculovirus vector, purified by affinity chromatography with the FLAG mAb, and then used to raise highly specific anti-Grb10 antibodies. When used to probe immunoblots of total protein extracts from COS-1 or HeLa cells, the antibodies recognize two major bands with apparent electrophoretic mobilities of 72 and 75 kDa (Fig. 1A), which probably result from Grb10 splicing variants, of which at least four are known to exist in human cells (5, 11, 34, 43). The two close homologues of Grb10, Grb7 and Grb14, with apparent electrophoretic mobilities of 58–65 kDa (3, 10), are not recognized by this serum. We also probed Triton-soluble extracts from a variety of human cell lines (Fig. 1B). Interestingly, the ratios between the two major immunoreactive bands varied according to the lineage of the hematopoietic cells. Cells of the erythroleukemia (K562) and T-cell (Jurkat, RPMI 8402, Peer) lineages had more of the faster migrating form, whereas the slower migrating form was more abundant in the B-cell derived Daudi cells. The U937 and HL-60 cell lines expressed the faster migrating form exclusively.

Immunolocalization of Endogenous and Overexpressed Grb10—Serum and immunoglobulins that were affinity-purified on a maltose-binding protein-Grb10 resin were used to detect the intracellular localization of endogenous Grb10 in COS-1 and HeLa cells. As seen in Fig. 2B, most of the endogenous Grb10 is localized to a number of large vesicular struc-
turedes that were identified as mitochondria by co-localization with a mAb that recognize the cytochrome oxidase subunit I (CoxI) (Fig. 3A). Labeling is also seen on the nuclei and, to a lower extent, in the cytoplasm, but reaction with preimmune serum has shown these to be nonspecific signals. Mitochondrial localization was also observed in HeLa cells, the original source of the hGrb10ζ cDNA, although lower expression levels of endogenous Grb10 (Fig. 1A) and a much higher background made interpretation of its localization more difficult (Fig. 2, E and F). In both cell lines, a 2-min treatment with 10% serum (not shown) or 100 ng/ml of IGF-I (Fig. 2C) induced the relocation of a small proportion of the endogenous Grb10 to the plasma membrane where it can theoretically interact with activated receptors. This translocation is very transient and is generally not observed 5 min after hormone treatment (not shown). After 30 min, some cells show a punctuate staining, which does not co-localize with the CoxI mitochondrial marker (Fig. 2D and not shown). Because this latter localization was observed only in a minority of cells, we did not study it further. Serum treatment of COS-1 cells induces the formation of actin-rich membrane ruffles, and, as shown in Fig. 3, these also became a site of Grb10 localization. Finally, Fig. 4 shows that overexpression of a FLAG-tagged hGrb10ζ in either COS-1 or HeLa cells results in its mislocalization to the cytoplasm. Identical results were obtained with GFP-Grb10 fusions. A small proportion of the Grb10 protein is still translocated to the plasma membrane shortly following serum treatment (not shown).

Cell Fractionation—To confirm the immunocytochemistry results, HeLa S3 cells were lysed in isotonic buffer and fractionated by differential centrifugation. The MP is enriched in mitochondria, peroxisomes, and large vesicles from the Golgi and endoplasmic reticulum. The plasma membrane and most of the endoplasmic reticulum/Golgi proteins were found in the high speed pellet, whereas cytosolic proteins are found in the high speed supernatant. Most of the endogenous Grb10 is found in the MP fraction (Fig. 5A). Interestingly, unlike attached HeLa cells that express equal amounts of the 72- and 75-kDa hGrb10 variants, the suspended HeLa S3 cells express mostly the 72-kDa variant. Furthermore, overexposing the

**Fig. 2.** Immunolocalization of endogenous Grb10 in cultured cells. COS-1 (A–D) and HeLa cells (E and F) probed with either preimmune serum (A and E) or affinity-purified anti-Grb10 (B–D and F). Cells were starved (B, E, and F) or treated with 100 ng/ml IGF-I for 30 min (D). Magnification is 1000×. Arrows show localization to the plasma membrane (C) or extra-mitochondrial vesicles (D).

**Fig. 3.** Co-localization of endogenous Grb10 with mitochondria and membrane ruffles. Starved COS-1 cells were treated with 10% FBS for 30 min and probed with anti-Grb10 serum (green) along with either CoxI mAb (A) or rhodamine-phalloidin (B, red). The right-hand panels show an overlap of the green and red images. Sites of co-localization appear yellow. Membrane ruffles are marked with white arrows. Magnification is 1000×.
Grb10 immunoblot revealed a band of 68 kDa mobility that is restricted to the soluble fraction and whose size is consistent with the hGrb10β splice variant. Grb10 was also detected in purified mitochondria-enriched heavy membrane fractions, caused enough damage to the mitochondrial membranes to release most of the Grb10 and Raf-1 proteins to the soluble fraction even though the fractionation of intramitochondrial proteins, such as CoxI, was maintained (Fig. 5C). Finally, we further purified mitochondria by centrifugation of the MP fraction through a discontinuous sucrose gradient. Fig. 6 shows that endogenous Grb10 can easily be detected in these highly purified organelles.

We next determined the nature of the Grb10-mitochondria interaction. Purified mitochondria were pelleted and resuspended in either isotonic buffer or a highly alkaline (pH 11.5) solution. The latter breaks open the organelles into linear membrane sheets, which can then be purified by high speed centrifugation (48). As shown in Fig. 6, the alkali treatment induces a relocalization of Grb10 from the pellet to the soluble fraction indicating that it is not a transmembrane protein. As expected, CoxI remained in the pellet fraction, whereas Raf-1 showed a distribution identical to Grb10 (not shown).

**DISCUSSION**

We demonstrate that, unexpectedly, the intracellular localization of the adapter protein Grb10 is mostly mitochondrial. Depending on growth conditions, a small proportion of Grb10 was also found on the plasma membrane as well as membrane ruffles. These observations were made possible by the production of a new highly specific antibody. Because of the low intensity of the specific labeling, we also probed our cells with preimmune sera from the same animal that was used for the production of the Grb10 antibodies. This control confirmed the nonspecificity of the nuclear and cytoplasmic fluorescence.

Mitochondrial localization of Grb10 was confirmed by cell fractionation experiments showing that most of the endogenous Grb10 is found in the mitochondria-enriched heavy membrane fraction. Grb10 was also detected in purified mitochondrial...
dria that were isolated though a discontinuous sucrose gradient. The population of Grb10 that is found in the light membrane fraction most probably represents those molecules that localize to the plasma membrane and membrane ruffles. The latter are the site of localization for many other signaling molecules involved in regulating the mitogenic MAP kinase pathway, most significantly Ras and Grb2 (49, 50). Our experiments further corroborate several reports that suggested the existence of a significant pool of Raf-1 kinase on the mitochondria (24, 26, 27, 51). The association of endogenous Grb10, and especially Raf-1, with the mitochondria is easily lost under harsher cell disruption conditions, which explains contradictory results from other laboratories (5, 26, 52).

Grb10 lacks any apparent mitochondrial localization sequence. This, together with the fact that overexpressing the protein leads to its mislocalization to the cytoplasm, and its peripheral association with membranes suggests that it uses a relatively low abundance lipid or protein as an anchor. One possible candidate for such an anchor is Raf-1 itself, which can be targeted to the mitochondria by Bcl-2 (26). This hypothesis was discounted because we estimate that there are approximately the same number of Grb10 and Raf-1 molecules per cell, whereas only about 10% of total Raf-1 proteins co-localize with endogenous Grb10 (Fig. 5). However, both proteins were easily co-immunoprecipitated following partial purification of the mitochondria, thus demonstrating that they indeed do interact in vivo. The affinity of Grb10 for mitochondrial Raf-1 is increased following activation of this kinase by ultraviolet light. This result is consistent with the relative co-localization of the Grb10-binding site with the Ras-binding domain of Raf-1. Studies of Ras-Raf interactions have shown this domain to be relatively inaccessible when the kinase is inactive (20, 28, 55).

The interaction of Grb10 with the anti-apoptotic mitochondrial Raf-1 kinase and our earlier results on the pro-apoptotic effects of Grb10 mutants (15) suggest that Grb10 could be used to regulate programmed cell death by modulating the activity of mitochondrial Raf-1. We thus propose that one of the functions of Grb10 is to serve as a link between plasma membrane receptors and the apoptosis-controlling complex on the mitochondrial outer membrane probably in collaboration with the phosphatidylinositol 3-kinase/Akt pathway, which plays a role in mediating the anti-apoptotic activity of the insulin and IGF-I receptors (56, 57). The phosphatidylinositol 3-kinase/Akt path-

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3 A. Nantel and D. Y. Thomas, unpublished data.

### FIG. 7. Two-hybrid analysis of Grb10-Raf-1 interaction.

Fragments of the Raf-1 kinase were fused in frame with the LexA DNA-binding protein and tested in an “interaction trap” assay with an acidic activator domain on its own or fused with the Grb10 SH2 domain. Successful interaction conferred the ability to grow in the absence of leucine, which is indicated by the + or ++ symbols, depending on the growth rate of the colonies. Not shown are Raf-1 constructs 1–254, 1–212, 1–138, 1–55, and 55–138, which exhibited high background levels.

### FIG. 8. Co-immunoprecipitation of endogenous Grb10 and Raf-1.

A, Grb10 immunoblot of mitochondrial protein extracts immunoprecipitated either with anti-Raf-1 mAb, preimmune serum, or anti-Grb10 serum. In the control lane, 10% of the extract used in the immunoprecipitations was loaded. B, Grb10 (top panel) or Raf-1 (middle panel) immunoblots of mitochondrial proteins extracts purified from starved or UV-treated cells and immunoprecipitated with a Raf-1 mAb. The bottom panel shows the amount of phosphorylated glutathione S-transferase-ERK1 following an immunocomplex kinase assay of the Raf-1 immunoprecipitates. The endogenous activity of the glutathione S-transferase-MEK1 kinase was estimated in a reaction that omitted the immunoprecipitate (control lane).
way has recently been shown to affect the phosphorylation state of both Grb10 and mitochondrial Raf-1 (27, 34).

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