ELSEVIER

Biochimica et Biophysica Acta 1371 (1998) 185–198

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Novel fluorescence membrane fusion assays reveal GTP-dependent fusogenic properties of outer mitochondrial membrane-derived proteins

Jorge D. Cortese *, Laura A. Voglino [1], Charles R. Hackenbrock

Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7090, USA

Received 14 October 1997; accepted 20 October 1997

Abstract

We have shown that fusion of small unilamellar vesicles (SUV) with outer mitochondrial membranes occurs at physiological pH [Cortese et al., 1991, J. Cell Biol., Vol. 113, 1331–1340]. The proteins driving this process could be involved in mitochondrial membrane fusion, which is presently poorly understood. In this study, we release from rat liver mitochondria a soluble protein fraction (SF) that increases fusion at neutral pH measured by membrane fusion assays (MFAs). Since this fusogenic activity was specifically enhanced by GTP, we separate SF by GTP affinity chromatography into: i) a flow-through subfraction (G1) containing numerous proteins with low GTP affinity; and ii) a subfraction (G2) which may contain GTP-binding proteins. A novel array of MFAs is developed to study the fusogenic properties of these fractions, measuring the merging of membranes (membrane-mixing) or the mixing of intravesicular aqueous contents (content-mixing). The MFAs use: a) SUV/large unilamellar vesicles, lacking mitochondrial membranes; b) SUV/mitochondria, reconstituting membrane–mitochondrial interactions; and c) mitochondria/mitochondria, mimicking mitochondrial fusion. The results indicate that: i) G1 contains GTP-independent, in vitro fusogenic proteins that are not sufficient to induce mitochondrial fusion; and ii) G2 contains GTP-dependent proteins that stimulate mitochondrial fusion at

Abbreviations: BCECF, fluorescent form of BCECF-AM; BCECF-AM,2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; CM, content-mixing; CME, fluorescent form of CMEDA; CMEDA, 5-chloromethyleosin diacetate; FITC-BSA, fluorescein-5-isothiocyanate-labeled bovine serum albumin; G1, subfraction from SF pool obtained as a flow-through in GTP agarose chromatography; G2, subfraction from SF pool retained in GTP agarose chromatography; H_{300} medium, 300 mOsm solution composed of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4) and 0.05% fatty acid-free bovine serum albumin (BSA); LUV, large unilamellar vesicles; MFA(s), membrane fusion assay(s); Mitochondria/mitochondria MFA, MFA that detects fusion between mitochondrial populations; MM, membrane-mixing; OMM, outer mitochondrial membrane; R_{18}, octadecylrhodamine; RCR, respiratory control ratio, defined as the ratio between oxygen consumption of mitochondria in respiratory state 3 (in the presence of substrate and ADP) and respiratory state 4 (in the presence of substrate alone); RET, resonance energy transfer; RSQ, relief of self-quenching; SUV, small unilamellar vesicles; SF, soluble protein fraction derived from OMM; SUV/LUV MFA, MFA that detects fusion between SUV and LUV; SUV/mitochondria MFA, MFA that detects fusion between SUV and mitochondria

* Corresponding author. Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, 117 C Taylor Hall, CB# 7090, Chapel Hill, NC 27599-7090, USA. Fax: +1-919-966-1856; E-mail: jcortes@med.unc.edu

[1] Present address: Department of Cell Biology, Duke University, 402 Sands Bldg., P.O. Box 3011, Durham, NC 27710, USA.

PII S0005-2736(97)00266-6

neutral pH. The MFAs described here could be used to monitor the isolation of active proteins from these subfractions and to define the mechanism of intermitochondrial membrane fusion.

Keywords: Guanine nucleotide; Liposome; Liver mitochondrion; Resonance energy transfer; Mitochondrial fusion; Fluorescence

1. Introduction

Mitochondria exhibit elongated or spherical shapes in many cell types, thus its name from the Greek *mitos*: 'thread' and *kondria*: 'bead' [1]. From early microscopic observations of mitochondrial morphology [2] and motility [3,4], to modern studies using video-enhanced and fluorescence microscopy [5–7], we have come to envision mitochondria as a cellular structure with the ability to fuse and fragment. Mitochondrial fusion occurs during cell development [8,9] and in several disease states that produce liver megamitochondria [10]. It has also been postulated to explain fast redistribution of mitochondrial DNA in HeLa cells [11], DNA exchange between organelle compartments [12,13] and mitochondrial genetic mixing induced by plasmids found in plasmodium strains [14,15]. The formation of a single yeast mitochondrion [16,17] might be also a result of protein-mediated membrane fusion and yeast proteins relevant to mitochondrial morphology changes have been detected by mutant analysis [18,19]. In spite of all these observations and of many others with biological systems where mitochondrial fusion has been detected or postulated [20–23], mitochondrial fusion is generally viewed as a spontaneous, unregulated process.

Although progress is being made in understanding the relationship between mitochondrial inheritance and mitochondrial fusion in yeast [18,19,24], it is lacking in mammalian cells, where different signalling pathways may be acting to regulate the process. It is also unclear how mitochondrial fusion helps to maintain the structure of mitochondria through cycles of cell division, or increases the redistribution of soluble ions, genetic material and membrane potentials [25,26] between mitochondria subjected to different microenvironments. Disruption of this dynamic mitochondrial system, sometimes known as 'reticulum mitochondriale' [27], are apparent as alterations of morphology, collectively named mitochondrial pleomorphism [10]. Uncovering the underlying biochemical mechanism for mammalian mitochondrial fusion can thus bring a variety of physiological and pathological conditions to a new understanding and possibly to genetic manipulation. In this report, we have undertaken the first steps towards identifying the proteins responsible for rat liver mitochondrial fusion.

During the development of a liposome-mediated, low pH-based method to deliver high molecular weight fluorescence probes into the intermembrane space of intact mitochondria [28–30], we detected a component of membrane fusion at neutral pH that could be mediated by outer mitochondrial membrane (OMM) proteins. The membrane fusion assay (MFA) used [28–30] was limited to measuring membrane- and content-mixing (MM and CM, respectively) between small unilamellar vesicles (SUV) and mitochondria, which may only partially represent the molecular events occurring during intermitochondrial fusion. Thus, it was necessary to develop additional MFAs to test all aspects of the process of mitochondrial fusion. This paper presents such an array of mitochondrial MFAs. We developed a simple, mitochondria-free MFA measuring membrane fusion between SUV and large unilamellar vesicles (LUV) and a MFA resembling the fusion between mitochondria, where MM between outer membranes or CM between mitochondrial matrices can be monitored continuously. Using these MFAs, we now show that a solubilized OMM protein fraction named SF stimulated membrane fusion (i.e., is fusogenic) and that GTP enhances the effect. Soluble subfractions derived SF through GTP affinity chromatography have a differential behavior under these MFAs: the subfraction that does not bind to GTP affinity columns contains proteins that enhance intermitochondrial binding and the protein pool retained by the GTP-binding column contains proteins that stimulate GTP-dependent mitochondrial fusion.

2. Materials and methods

2.1. Materials

Fatty acid-free bovine serum albumin (BSA), EDTA, GDP, guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS), GTP, guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS), D-mannitol, sodium dodecyl sulfate (SDS), succinate, sucrose and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO). Asolectin was purchased from Associated Concentrates (Woodside, L.I., NY), digitonin from Calbiochem (La Jolla, CA) and Hepes was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were of the highest purity available commercially. Sephadex G-25 and G-200 were obtained from Pharmacia (Uppsala, Sweden), GTP-ActiGel-ALD was from Sterogene Bioseparations (Arcadia, CA) and Centriprep-10 concentrators (10 kD-molecular weight cutoff) from Amicon (Beverly, CA). BCECF-AM, CMEDA, F_{16}, F_{18} and R_{18} (chloride salt) were purchased from Molecular Probes (Eugene, OR). FITC-BSA was prepared as described [28,31], or purchased commercially (Molecular Probes).

2.2. Mitochondria-derived preparations

Liver mitochondria were isolated from male Sprague–Dawley rats [28,32] and then resuspended in isosmotic H_{300} medium. Respiratory control ratios (RCR) were used as an index of mitochondrial intactness, measured polarographically with a Clark oxygen electrode according to Cortese et al. [28,33]. RCR values for our preparations (succinate/ADP system; [28]) were in the range 5.0–8.0, indicative of highly purified mitochondria.

Our goal of identifying mitochondrial proteins involved in mitochondrial fusion cannot be easily accomplished by direct extraction from intact mitochondria. We have been able to use high ionic strength/digitonin to perturbate extrinsic protein–membrane interactions, releasing a soluble fraction that may contain OMM-derived fusogenic proteins. Isolated rat liver mitochondria obtained from eight rats (5 mg/ml) were kept on ice for 15 min in an incubation buffer containing 75 mM KCl, 110 mM mannitol, 35 mM sucrose and 2 mM Hepes (pH 7.4). Digitonin was then added at a concentration below that needed to induce release of intermembrane space enzyme markers (1 mg digitonin/100 mg mitochondria; [32]). The sample was diluted (1:15) and spun for 15 min at $9770 \times g$ (4°C), the supernatant was collected and the wash step repeated. Pooled supernatants were then diluted (1:4) and centrifuged for 1 h at $104{,}000 \times g$ (4°C), to remove any membrane fragments. The resulting soluble fraction (SF) was concentrated to 2–4 mg/ml using a Centriprep-10 concentrator and loaded into a GTP-agarose column (GTP-ActiGel-ALD). A protein mixture that does not bind GTP-agarose was eluted first at a 1 ml/min-flow rate with a buffer containing 10% glycerol, 0.1 mM EDTA, 0.15 mM $MnCl_2$ and 10 mM Hepes (pH 7.4). This first protein subfraction was named G1. Proteins bound to GTP-agarose at low ionic strength were eluted with a high ionic strength buffer containing 10% glycerol, 0.1 mM EDTA, 0.5 M KCl and 10 mM Hepes (pH 7.4). This second protein subfraction was named G2. These protein subfractions were concentrated to 2–4 mg/ml and kept on ice for use in experiments within 4 h. The conditions described above were based on Colombo et al. [34], modified to optimize separation at low protein concentration. The same elution pattern and sample composition were obtained if 1–5 mM GTP was used as eluant, but we eliminated GTP additions from our preparations, thus recovering G2 proteins in the GTP-unbound form. Protein composition was assessed by SDS-PAGE [35] and protein concentrations were measured using the MicroBCA protein assay [36]. Contamination of OMM-derived fractions with mitochondrial phospholipase A [37] could affect the results from MFAs. Within the sensitivity of the pH-based activity assay [38,39], we could not detect any phospholipase activity in SF or SF-derived fractions and a low level of activity (0.20 ± 0.05 U/mg mitochondrial protein with 5 mM Ca^{2+} at 37°C) was detected for mitochondria.

2.3. Fluorescence measurements

The intensity of fluorescence emission was monitored digitally using a Perkin-Elmer fluorescence spectrophotometer model 650-40 (Perkin-Elmer, Norwalk, CT) in the ratio mode at 15°C. The excitation wavelength was 490 nm for fluorescein-linked dyes (5-(*N*-hexadecanoyl) aminofluorescein or F_{16}, *N*-oc-

tadecyl-*N*-(5-(fluoresceinyl)) thiourea or F_{18} and FITC-BSA), 500 nm for BCECF (fluorescent form of 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; BCECF-AM), 560 nm for octadecylrhodamine (R_{18}) and 525 nm for CME (fluorescent form of 5-chloromethyleosin diacetate; CMEDA); using slit widths of 5 nm. When mitochondria were used in our membrane fusion assays, excitation of fluorescein-linked dyes and BCECF was moved down to 468 nm, thus minimizing errors introduced by light scattering of mitochondria [28]. The intensity of fluorescein emissions from fluorescein-linked dyes (520 nm), BCECF (525 nm), R_{18} (590 nm) and CME (565 nm), were all collected using slit widths of 10 nm. Quinine sulfate in 0.1 M H_2SO_4 was used to monitor changes on the efficiency of fluorescence intensity, before and after each experiment. Controls for turbidity were included when appropriate and inner filter effect corrections were those of Geren and Millet [40].

2.4. Membrane fusion assays (MFAs)

2.4.1. Assays using relief of self-quenching (RSQ)

We [28] have developed MFAs that measure the merging of membrane bilayers (MM; [41–43]) and the mixing of intravesicular contents (CM; [44,45]) during membrane fusion. The assays are based on the increase of apparent fluorescence that fluorophores exhibit when they are significantly diluted from a concentrated compartment (i.e., the phenomenon known as relief of self-quenching or RSQ).

Asolectin SUV labeled with R_{18} or encapsulating FITC-BSA were prepared as previously described, including gel filtration chromatography to eliminate fluorescence contributions from non-encapsulated material [28]. In our hands, asolectin SUV fuse readily with mitochondrial membranes at pH = 6.5 [28–30,46] and provide an economical means to substitute for synthetic phospholipids, when a large number of assays have to be carried out. SUVs prepared from synthetic lipids (molar ratio 4:1 of dioleoyl-phosphatidylcholine:dioleoyl-phosphatidylethanolamine) were also used in parallel assays, giving similar dequenching profiles (not shown). RSQ-based CM- or MM-SUV/mitochondria MFAs were carried out with SUV labeled with self-quenching concentrations of membrane-incorporated R_{18} (70 μM; MM) or soluble FITC-BSA (25 mg/ml; CM), mixed with unlabeled mitochondria (4.5 mg phospholipid/100 mg mitochondria). Lipid concentration was estimated by inorganic phosphate analysis [47]. Mitochondrial concentration was kept below 0.5 mg/ml, to avoid light scattering artifacts and H_{300} medium without BSA was used for all dilutions. These MFAs were carried out at 15°C, a condition that keeps mitochondria intact for $\approx$ 1 h [28].

We monitored the effects of RSQ through continuous or periodic measurements of fluorescence; proteins and effectors were added at appropriate times as described. These conditions maintain pH = 7.4 during 1 h-incubations; pH was slowly lowered to trigger fusion, using calibrated amounts of 0.1 M Hepes buffer (pH 5.0). The first recorded point is taken as time zero fluorescence (F_0) and fluorescence at infinite dilution (F_∞) estimated by serial dilution with 0.1% Triton X-100. Normalized RSQ fluorescence, i.e., $[F-F_0]/[F_\infty-F_0]$, a value independent of probe concentration, is used for interexperimental comparison. This is analogous to show results as a percentage of maximal attainable fusion. Results of all MFAs shown here are the average of at least three measurements and they represent the trend from three different mitochondrial preparations.

We also developed a MFA where SUV fuse with large unilamellar vesicles (LUV). LUV were prepared by repeated extrusion through polycarbonate filters [48] of a suspension containing 0.8 mg asolectin phospholipid per ml of H_{300} medium without BSA. SUV labeled with self-quenching concentrations of R_{18} (MM-SUV/LUV MFA) or FITC-BSA (CM-SUV/LUV MFA) were prepared as described above. In the assay cuvette, a 30:1 ratio of mg LUV phospholipid:mg SUV phospholipid was kept. The assay was carried out at 15°C, with a LUV concentration of 1.2 mg/ml and normalized RSQ fluorescence calculated as described above, including serial dilutions in 0.1% Triton X-100 to determine the fluorescence at infinite dilution (F_∞). Medium pH was decreased with buffer as described above.

To carry out a MM-mitochondria/mitochondria MFA, mitochondria (0.25 mg/ml) was labeled with R_{18} (240 μM) for 15 min on ice (H_{300} medium without BSA), extensively washed (800,000-fold effective dilution) to remove soluble R_{18} [28] and exposed to unlabeled mitochondria (12 μg/ml of

R_{18}-labeled mitochondria plus 120 μg/ml unlabeled mitochondria). RSQ from R_{18} was measured as described above. The R_{18}-labeled mitochondria preserved 80–95% of their RCR [28]. Also, since using 0.5 mg/ml BSA did not affect RSQ values measured by this MFA (not shown), unspecific protein-mediated lipid transfer such as that carried out by BSA is not likely to explain our results. Residual digitonin from the extraction protocol does not affect the RSQ MM-mitochondria/mitochondria MFA. Adding digitonin at concentrations that are 20-fold higher than those calculated for the cuvette assay (i.e., 0.01 mg/ml) had no effect on RSQ from mitochondrial controls, or from samples with the addition of SF, G1 or G2 subfractions (250 μg/ml; not shown).

2.4.2. Assay using resonance energy transfer (RET)

A CM-mitochondria/mitochondria MFA was developed using RET between matrix-entrapped fluorescence probes. BCECF was used as a RET donor [49–51] and CME as a RET acceptor [30,52,53]. Mitochondria (25 mg/ml in H_{300} medium without BSA) were labeled with BCECF-AM (52 μM) or with CMEDA (60 μM) for 45 min at 15°C. BCECF remains entrapped into the mitochondrial matrix after this incubation [50]. A further incubation for 45 min at 15°C with media containing respiratory substrate (H_{300} medium with 1.5 mM K_2PO_4, 2.5 mM Na-succinate, 1.5 mM $MgCl_2$ and 0.5 mM Na_2–EDTA), was necessary to obtain covalent linkage of CME to matrix proteins, thus entrapping the probe into mitochondria. The two probes used were retained into the mitochondrial matrix, even after extensive washes (800,000-fold final dilution after serial dilutions). RCR of CMEDA- and BCECF-AM-treated samples was 75–90% of values for untreated mitochondria. The assay proceeds by monitoring BCECF emission (donor quenching method; [30]). Controls using donor (D)- or acceptor (A)-labeled mitochondria exposed to unlabeled mitochondria are also included. An assay using BCECF-labeled plus unlabeled mitochondria gave the unquenched donor intensity (F_D), which was compared with a parallel assay run using CME-labeled (0.1 mg/ml) and BCECF-labeled mitochondria (0.4 mg/ml) to obtain the quenched donor intensity (F_{DA}). RET efficiency (E_{RET}) was calculated as $E_{RET} = [1 - (F_D/F_{DA})]$. The maximum attainable RET (taken as 100%; RET_{max}) was estimated using hypotonically-lysed mixtures of mitochondria previously labeled with CME and BCECF. Based on the above definition, the RET-based percentage of content-mixing was estimated as: $100 * [E_{RET}/RET_{max}]$. A calibration curve was constructed using mixtures of mitochondria labeled with BCECF-AM and CMEDA, lysed to bring both probes into contact. To carry out this 'mock' fusion assay, both probes were entrapped into the mitochondrial matrix as described above. Subsequently, mitochondria were hypotonically lysed (1:10 dilution with distilled water), pelleted and resuspended in H_{300} medium without BSA and the supernatant concentrated to obtain a 0.1 mg/ml protein solution. The estimated BCECF-CME's spectral overlap J was 72% of the value found for the fluorescein–rhodamine RET couple [30]. As only E_{RET} has to be measured to estimate intermitochondrial content-mixing, we have not characterized this RET couple to the point of obtaining statistically-ranged interprobe distances [30].

For the RET-based MM-mitochondria/mitochondria assay used in Fig. 3, two samples of mitochondria suspended at 0.5 mg/ml in H_{300} medium without BSA were labeled with either $R_{18} = 50$ μM or $F_{16} = 35$ μM for 15 min on ice. A 'mock' fusion calibration curve was constructed using mitochondria labeled with mixtures of R_{18} and F_{16}, showing that changes in RET are linear for the full 0–100% range of calculated MM percentage (not shown). Differences of more than 3% RET between samples can be detected with this assay.

3. Results

Membrane fusion assays (MFAs) that measure membrane- and content-mixing between SUV and mitochondria, based on exposing isolated rat liver mitochondria to SUV loaded with fluorescence probes for 1 h at pH 7.4 and then decreasing the pH to 6.5 to prompt probe delivery, were characterized by a large increase in RSQ (Fig. 1). Mitochondria were not structurally or functionally damaged by this procedure [28]. These fluorescence MFAs revealed that a component of fusion was always present at neutral pH (Fig. 3 of [28]), suggesting that proteins present in the OMM could be responsible. We thus decided to separate from the OMM protein fractions capable of enhancing this effect. Experimentation led to a solu-

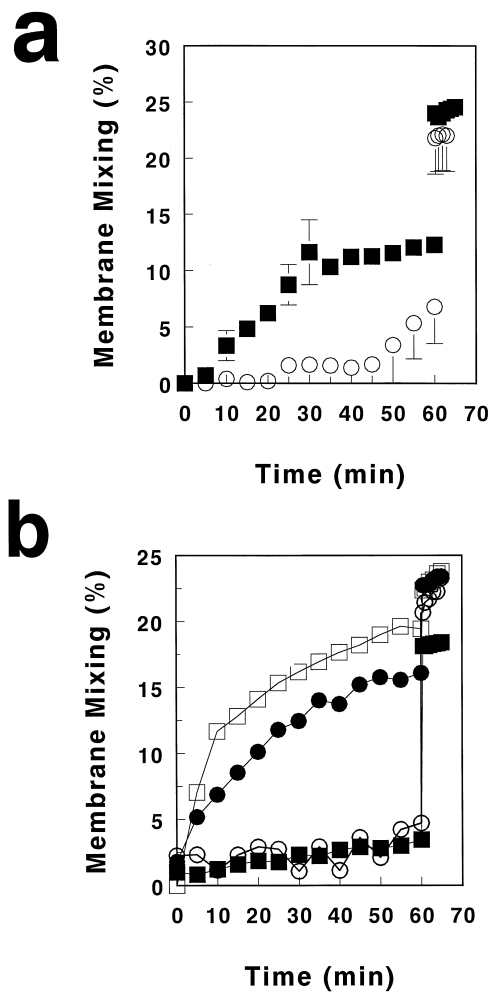

Fig. 1. A mitochondrial membrane fusion assay (MFA) shows nucleotide-dependent effects of an outer membrane-derived, soluble protein fraction (SF). The RSQ-based MM-SUV/mitochondria MFA was carried out. Mitochondria were incubated in H_{300} medium without BSA (0.2 mg/ml; 15°C), followed by the addition (time equal to zero) of: (a) R_{18}-labeled SUV (mitochondrial control; ○), R_{18}-labeled SUV plus SF fraction (250 μg/ml; ■) and (b) Same MFA as in (a), with addition of R_{18}-labeled SUV (mitochondrial control; ○), R_{18}-labeled SUV plus 0.5 mM GTP (●), R_{18}-labeled SUV plus 0.5 mM ATP (■) and R_{18}-labeled SUV plus SF (250 μg/ml) and GTP (200 μM; □). After a 60 min-incubation at pH 7.4, pH was lowered to 6.5 by addition (5% v/v) of 1 M Hepes pH 5.0 and measurements taken every 15 s.

ble fraction named SF (see Section 2) which increased the neutral pH component of membrane fusion (RSQ-based MM-SUV/mitochondria MFA; Fig. 1a). The low pH-induced fusion was not affected, suggesting that only the efficiency of fusion at neutral pH was altered, not the maximal attainable fusion. Membrane fusion at neutral pH appears to be specifically modified by nucleotides (Fig. 1b). Addition of ATP did not modify substantially the fusion profile, while addition of GTP increased the neutral pH component. Joint addition of SF and GTP increased membrane fusion at neutral pH to near the maximum measurable on this MFA.

Using a mitochondria-free MFA (SUV/LUV), we showed that the GTP dependence of the effect of SF on mitochondrial membrane fusion requires the presence of intact mitochondria. Addition of SF to the SUV/LUV MFA increased fusion at neutral pH (Fig. 2) however, addition of 1 mM GTP did not further increase fusion at neutral pH. Content-mixing changes parallel membrane-mixing changes detected by this assay (not shown). The limited increase of membrane-mixing by the addition of SF at neutral pH suggests that proteins present in SF could cause vesicle membrane fusion even at neutral pH and in the absence of mitochondrial membrane proteins.

A further test for the effects of the SF protein pool was carried out (Fig. 3), using a resonance energy transfer (RET) assay that is exceedingly sensitive to any amount of intermembrane lipid-mixing, whether caused by fusion or intimate membrane contact. The

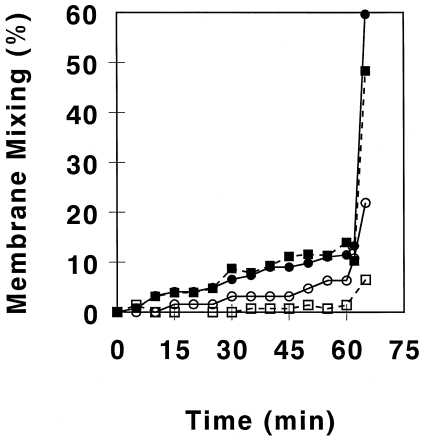

Fig. 2. Effect of SF and GTP on a mitochondria-free MFA. The RSQ-based, MM-SUV/LUV MFA described was used. LUV were incubated in H_{300} medium without BSA, followed by the addition at time equal to zero of: (a) R_{18}-labeled SUV (—○); (b) Same as in (a), plus 1 mM GTP (—□); (c) Same as in (a), plus 600 μg/ml SF (—●); and (d) Same as in (c), plus 1 mM GTP (– – –■).

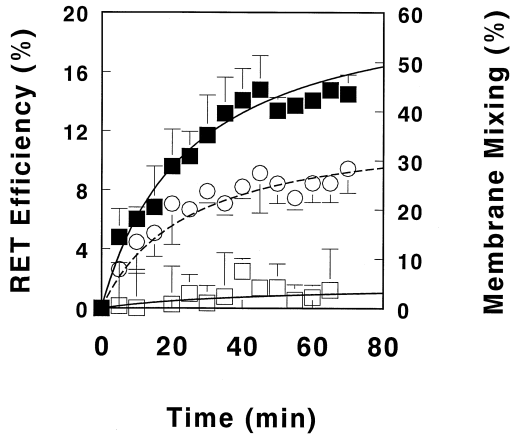

Fig. 3. The effect of SF on a resonance energy transfer (RET)-based intermitochondrial MM-MFA. RET between mitochondrial populations (0.5 mg/ml in H_{300} medium without BSA) labeled with R_{18} and F_{16} was measured as described for: (a) untreated mitochondria (○); (b) mitochondria after the addition of the SF fraction (250 μg/ml; ■); and (c) mitochondria washed three times with 145 mM KCl, 2 mM Hepes pH 7.4 (□). Percentage of membrane-mixing was calculated by comparison with a calibration assay.

RET-based MM-mitochondria/mitochondria MFA detects RET change over time (Fig. 3), as the probes slowly equilibrate between mitochondrial membranes. Also, as occurred with the MM-SUV/mitochondria and MM-SUV/LUV MFAs, addition of SF caused an increase in the rate of MM over a 1 h-incubation period at neutral pH. When mitochondria were pretreated by washing at high, physiological ionic strength (125 mM KCl, 2 mM Hepes pH 7.4), there was very little time-dependent change in RET. This treatment also decreased membrane fusion between SUV and mitochondria (not shown). We used physiological ionic strength washes to substitute the digitonin/ionic strength treatment, since detergents perturbate this MFA. Physiological ionic strength removes most of the components of the SF pool (not shown). High ionic washes did not affect mitochondrial function; RCR from washed, labeled mitochondria were 85–95% of control, intact mitochondria. Although not conclusively proving mitochondrial fusion, our results strongly suggest that proteins contained in SF can enhance intermitochondrial binding (see Section 4).

The GTP-dependent effect of SF on fusion between SUV and mitochondria (or LUV) indicates that this fraction may contain soluble GTP-sensitive proteins or subunits from transmembrane proteins involved in mitochondrial fusion. Thus, we separated the proteins contained in the SF protein pool using GTP-affinity chromatography. Two distinct protein fractions were obtained (Fig. 4a): the flow-through protein pool (G1) contains most of the proteins present in SF and the protein pool retained in the column

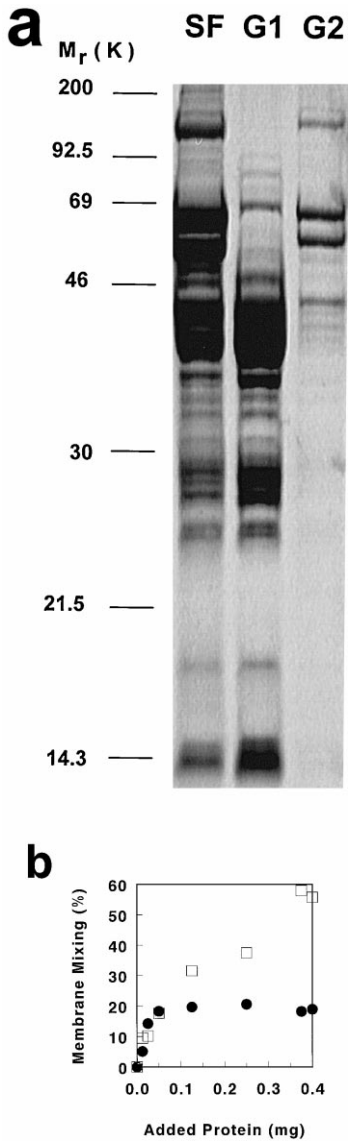

Fig. 4. Separation of the SF protein pool into fractions with differential fusogenic proteins. (a) Coomasie Blue protein staining of a 12.5% SDS-acrylamide gel. Molecular weights (in kDa) are shown in the left. SF, soluble protein pool derived from mitochondria; G1 and G2, fractions eluted from GTP-agarose. (b) MM-SUV/mitochondria MFA used in Fig. 1, with the addition of G1 (□) and G2 (●) in the amounts indicated in the *x*-axis.

(G2) has only a few distinctive bands. Both subfractions were tested using the MM-SUV/mitochondria MFA and the resulting titrations (Fig. 4b) show a differential behavior. G1 exhibits a linear, concentration-dependent effect on membrane-mixing, as expected from a non-specific fusogenic fraction that facilitates intermembrane contact between SUV and mitochondria. Within the same range of protein additions, the G2 subfraction leads to a saturation behavior and a plateau was reached with the addition of only 50 μg of G2 per cuvette assay.

We conducted several additional tests with the G1 and G2 subfractions (Fig. 5). Addition of G1 increases MM and CM in both the SUV/LUV and SUV/mitochondria MFAs (cf. Fig. 4b). This again suggests that this subfraction contains fusogenic proteins. Addition of G2 affected the SUV/LUV MFA to a lesser extent than G1. G2 increased MM in the SUV/mitochondria MFA, but did not affect the CM-SUV/mitochondria MFA. This indicates that G2 is inducing intermembrane contacts between SUV and

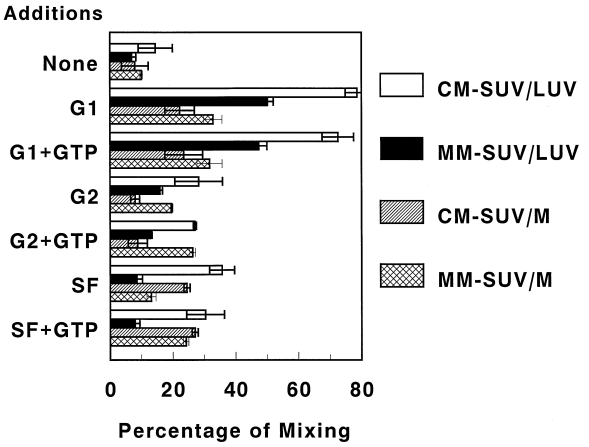

Fig. 5. Membrane fusion assays (MFAs) show differential fusogenic properties of OMM-derived protein fractions. The MFAs using relief of self-quenching (RSQ) of SUV labeled with an excess of soluble, fluorescent probes (MM with R_{18} and CM with FITC-BSA) described in Section 2 were carried out: (i) with mitochondria-free preparations (CM-SUV/LUV and MM-SUV/LUV); and (ii) with mixed vesicle–mitochondrial preparations (CM-SUV/mitochondria and MM-SUV/mitochondria). The assays were carried out at pH 7.4 without additions (None), or the samples were treated with: (a) G1 (250 μg/ml); (b) same as in (a), plus GTP (200 μM; G1+GTP); (c) G2 (250 μg/ml); (d) G2 same as in (c), plus GTP (200 μM; G2+GTP); (e) SF (500 μg/ml); (f) SF as in (e), plus GTP (200 μM; SF+GTP). Bar lines are indicated in the right.

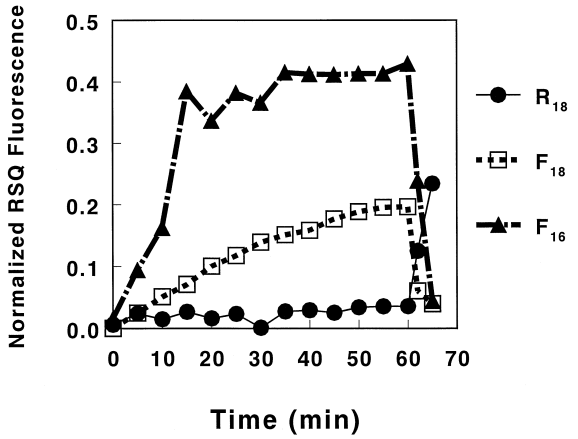

Fig. 6. Fluorescence probes that produce relief of self-quenching compatible with a MM-mitochondria/mitochondria MFA. The RSQ-based MM-mitochondria/mitochondria MFA described was carried out using R_{18} = 500 μM (——●), F_{18} = 500 μM (– – –□), or F_{16} = 500 μM (–·–▲) to label mitochondria. The MFA was started by the addition (time equal to zero) of labeled mitochondria to a 10-fold greater excess of unlabeled mitochondria (0.5 mg/ml final concentration). Before the MFA was carried out, mitochondria were washed twice with 145 mM KCl, 2 mM Hepes pH 7.4. After a 1 h-incubation at pH 7.4, the pH was lowered to 6.5 as described in Fig. 1.

mitochondria rather than fusion. GTP addition did not modify G2's effect on the CM- and MM-SUV/LUV MFA, suggesting that other OMM proteins are necessary for GTP-dependent effects. Addition of SF increased MM and CM in the SUV/mitochondria MFA (cf. Fig. 1a), but further addition of GTP (0.5 mM) only affected MM in this assay (cf. Fig. 1b). SF had less effect on these MFAs than G1 or G2, suggesting interactions between G1 and G2 proteins. Since both SF and G2 show GTP-dependent effects in MFAs, while G1 effects are GTP-insensitive, G2 might contain the protein or proteins that specifically participate in GTP-dependent events of membrane fusion.

A RSQ-based MFA was developed to assay intermitochondrial membrane-mixing. Although we have used R_{18} in another RSQ-based MM-MFA involving SUV [28], we validated here its use for mitochondrial membranes (Fig. 6). Use of fluorescein-linked probes at self-quenching concentrations was not optimal, as F_{16} and F_{18} showed large RSQ increases at neutral pH. This suggests collisional migration of F_{16} and F_{18} between labeled and unlabeled membranes, which will give a false positive in a RSQ MFA. The effect

appears at the high concentrations of these probes used in this RSQ MFA, but was not apparent at the concentrations used in the MM-MFA shown in Fig. 3. Fluorescein-linked probes are also pH-sensitive [28,29,49,51] and lowering pH to prompt membrane fusion decreases their fluorescence, obscuring any signal from low pH-induced RSQ (Fig. 6). Fluorescence from R_{18} is not pH-sensitive, showing a low RSQ signal at neutral pH and a small increase when pH was lowered, as expected from a dye that does not migrate between mitochondrial membranes through collisional events. R_{18} has a net positive charge and it may bind very strongly to the negatively charged mitochondrial membrane, while the negatively charged F_{16} and F_{18} would be easily removed from these membranes. Thus, R_{18} seems the appropriate choice for our RSQ MM-MFA involving mitochondrial membranes. Phospholipid-linked probes could not be efficiently incorporated into mitochondrial membranes without detergents or solvents that perturb mitochondrial function (not shown).

We used the RSQ MM-mitochondria/mitochondria MFA to determine the effect of G1 and G2 on intermitochondrial MM (Fig. 7a). Addition of G1 increased MM substantially, but the increase was GTP insensitive. G2 did increase MM, as with the MM-SUV/mitochondria MFA. Membrane-mixing increased further when GTP was added after a 1 h-incubation at neutral pH. We also tested the specificity of the guanosine nucleotide binding with the MM-mitochondria/mitochondria MFA (Fig. 7b). GTP stimulated MM on controls and G-2 treated samples and GTPγS only affected this MFA when added together with G2. GDP and GDPβS lacked significant effect on MM (see Section 4). Thus, in this mitochondrial MFA G2 showed its distinctive GTP-mediated MM increase, indicating that some component of G2 acts stimulating GTP-dependent mitochondrial membrane interactions. However, an additional CM-mitochondria/mitochondria MFA was necessary, to clarify if G1 and G2-dependent effects lead to mitochondrial fusion.

To measure intermitochondrial CM, we used probes that can be delivered into the mitochondrial matrix (see Section 2). BCECF-AM is irreversibly entrapped into the matrix through removal of its acetomethyl group by mitochondrial esterases [50]; CMEDA is activated and covalently linked to matrix

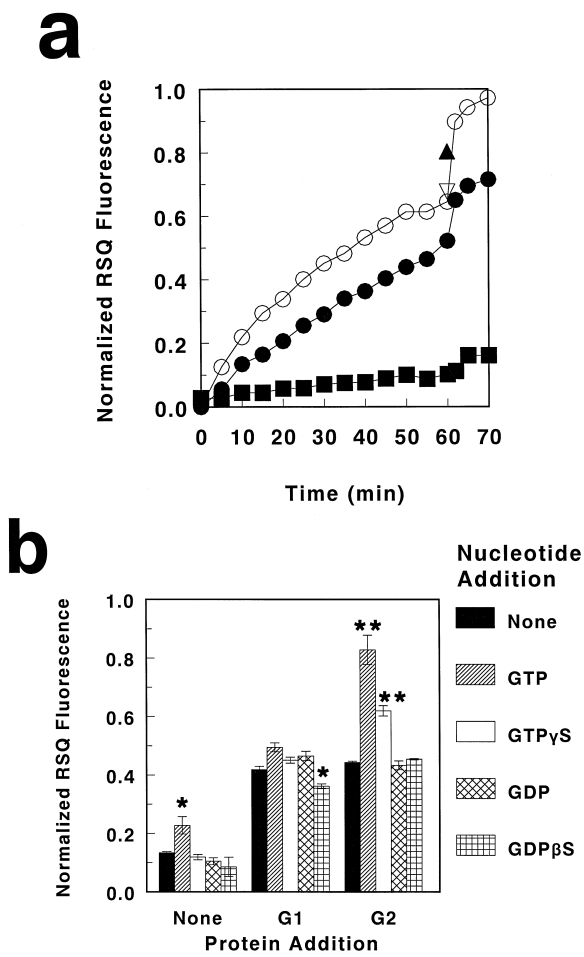

Fig. 7. Differential effect of SF-derived protein subfractions and GTP in a RSQ-based, MM-mitochondria/mitochondria MFA. (a) The assay was carried out with no additions (■), or with the addition of 50 μg/ml G1 (○), 50 μg/ml G2 (●), G1 plus 0.5 mM GTP (∇), G2 plus 0.5 mM GTP (▲). Incubations at pH = 7.4 and pH = 6.5 were as described in Fig. 6. (b) The MFA was carried out with no protein additions (None), or with the addition of 50 μg/ml G1 (G1) or 50 μg/ml G2 (G2). For each of these assays, samples were included without added nucleotide (None), or with the addition of 200 μM GTP (GTP), 100 μM GTPγS (GTPγS), 200 μM GDP (GDP), or 100 GDPβS (GDPβS). Four measurements were averaged in each condition and tested statistically to verify that differences with respect to samples without added nucleotide are significant. A positive result is indicated with * ($p = 0.05$) or ** ($p = 0.01$).

proteins [52,53]. The matrix-entrapped fluorescence forms of these probes (BCECF and CME) were used as RET donor and acceptor, respectively. We verified that the absorption spectra of BCECF and the fluorescence emission spectra of CME overlap significantly (Fig. 8a) and that both fluorescence emission spectra

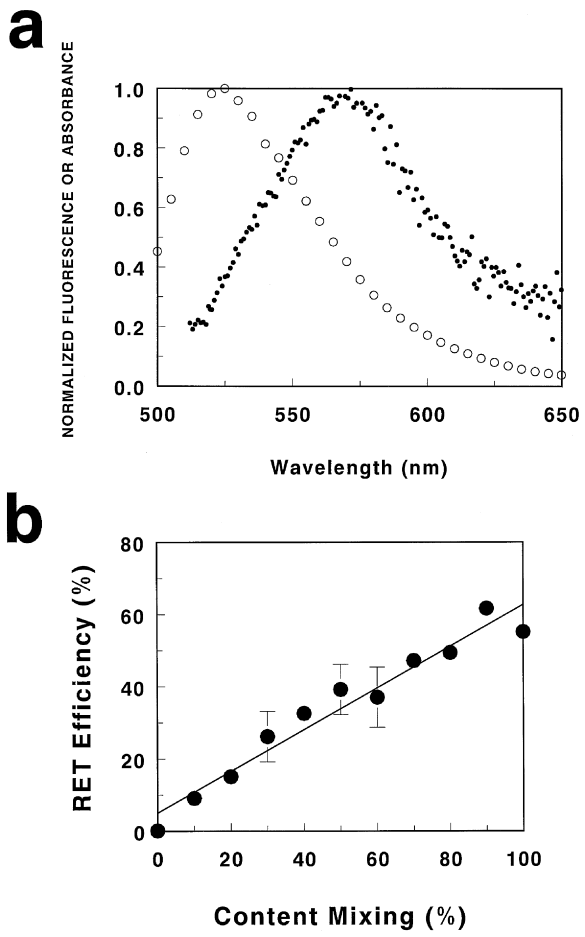

Fig. 8. Spectral calibration of the fluorescent forms of matrix-entrappable probes. (a) Spectral overlap between the normalized emission fluorescence spectra of BCECF (○) and the absorbance of CME (proportional to the excitation spectra; ●). Both probes were entrapped into mitochondria and the organelle later lysed as described in Section 2. (b) Mixtures containing different proportions of mitochondria labeled and lysed as in (a) were prepared. Measurements and calculation of RET efficiency described were carried out for BCECF-labeled plus unlabeled lysed mitochondria (F_A) and BCECF-labeled plus CME-labeled lysed mitochondria (F_{DA}). The 100% CM value was estimated with the lysate of a 50%–50% mixture of BCECF- and CME-labeled mitochondria.

are modified when the probes are brought together in solution, as expected from RET-coupled fluorophores [30]. We also carried out a 'mock' experiment using BCECF- and CME-labeled mitochondria, mixed in different proportions and hypotonically-lysed (Fig. 8b). RET increases proportionally to the percentage of probes mixed together. Mixing of these two probes in the mitochondrial matrix thus leads to a change in fluorescence consistent with RET.

Using this CM-mitochondria/mitochondria MFA, we studied the effects of the addition of G1 (Fig. 9a) or G2 (Fig. 9b) to intact mitochondria. There was a small degree of time-dependent CM without protein addition, up to about 10% RET efficiency (9% CM). Addition of G1 to this MFA inhibits this CM component to about 2.5% RET efficiency with 200 μg of

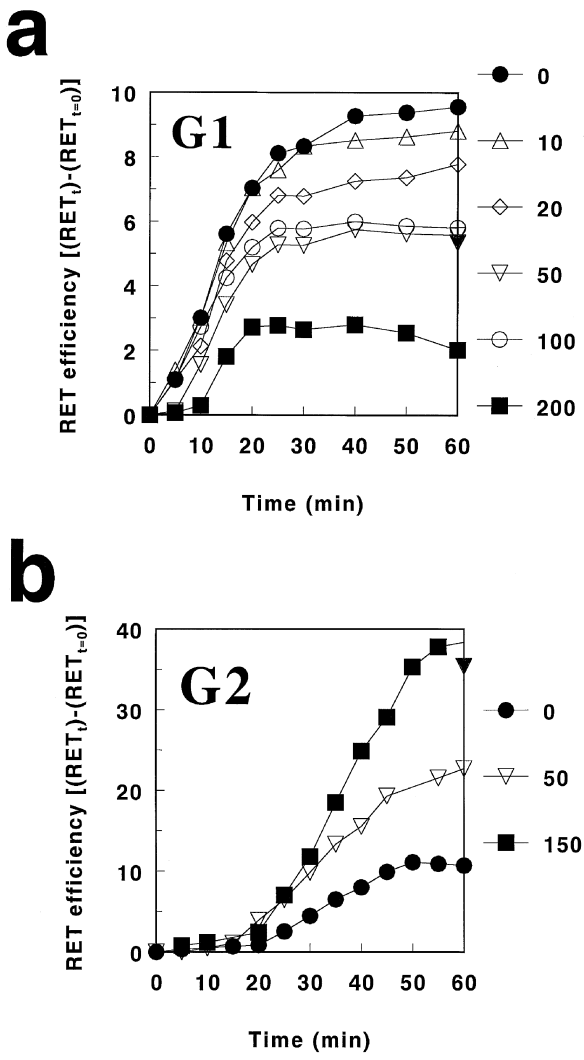

Fig. 9. Differential effect of SF-derived protein subfractions in a RET-based, CM-mitochondria/mitochondria MFA. The MFA was carried out for mitochondria exposed to various concentrations of: (a) G1, with concentrations in the range 0–200 μg/ml. An additional point with 50 ug/ml G1 and 200 μM GTP is shown (▼); (b) G2, with concentrations in the range 0–150 μg/ml. An additional point with 50 ug/ml G2 and 200 μM GTP is shown (▼). Data were represented as the difference between RET efficiency at a given time with respect to RET efficiency at time equal to zero. Symbols used are indicated in the right side of each plot.

Table 1
Membrane fusion assays (MFAs) applied to OMM-derived protein subfractions

Additions	+G1		+G2		+G1 + GTP		+G2 + GTP	
MFA	MM	CM	MM	CM	MM	CM	MM	CM
SUV/LUV	+++[a]	+++[a]	++[f]	++[f]	+++[k]	+++[k]	++[n]	++[n]
SUV/M	++[b]	++[c]	+[g]	−[h]	++[k]	++[k]	++[o]	−[o]
M/M	++[d]	−[e]	++[I]	++[j]	++[l]	−[m]	+++[p]	+++[q]

SUV/M, SUV/mitochondria; M/M, mitochondria/mitochondria.
[a]—Fig. 5.
[b]—Fig. 4b (increments proportional to G1 concentration added) and Fig. 5.
[c]—Similar to data shown in Fig. 4b (not shown).
[d]—Fig. 7.
[e]—Fig. 9a; addition of G1 inhibits CM.
[f]—Fig. 5.
[g]—Fig. 4b (saturable by addition of G2).
[h]—Similar to data shown in Fig. 4b (not shown).
[i]—Fig. 7a.
[j]—Fig. 9b.
[k]—Fig. 5; GTP had no additional effect over the addition of G1 alone.
[l]—Fig. 7a; GTP had no additional effect over the addition of G1 alone.
[m]—Fig. 9a; GTP had no additional effect over the addition of G1 alone.
[n]—Fig. 5; GTP had no effect over the addition of G2 alone.
[o]—Fig. 5.
[p]—Fig. 7; positive effect over the addition of G2 alone.
[q]—Fig. 9b; GTP had a positive effect over the addition of G2 alone.

G1 (Fig. 9a). Since G1 was able to induce CM and MM (an index of true membrane fusion) in the SUV/LUV and SUV/mitochondria MFA and lead to increases in the MM-mitochondria/mitochondria MFA (Fig. 7), this result shows that G1 is not sufficient to induce fusion when acting in a mitochondrion–mitochondrion complex. GTP did not affect the CM-mitochondrial MFA with added G1. Addition of G2 increases CM, up to 40% RET efficiency (35% CM) with 150 μg of G2 (Fig. 9b). Thus, the addition of excess G2 can stimulate mitochondrial fusion measured as CM and MM increments. Since G2 only stimulated the MM-SUV/mitochondria MFA, the presence of proteins others that those in G2 is required for intermitochondrial CM. As with the other MFAs, the effect of G2 on the CM-mitochondrial MFA was GTP sensitive (Fig. 9b). The results of the experiments carried out with all these MFAs are summarized in Table 1.

4. Discussion

Many studies in cellular fusion are carried out with one or two assays that detect fusion, commonly MFAs that use cell membrane ghosts or LUVs to measure membrane- or content-mixing [54–56], or complex biological assays where fusion is inferred from changes in a biological structure [57], biochemical activity [58], or a biophysical parameter [59]. For the study of mitochondrial fusion, additional assays are necessary to dissect putative receptor–activator–fusogenic protein complexes carrying out regulated organelle fusion. Ideally, these new MFAs should reconstitute a subset of the many interactions occurring between these proteins. For example, MFAs lacking mitochondrial membranes (and the putative proteinaceous receptor that participates in interorganelle recognition) will be sensitive to the effect of purely fusogenic components. These components will act directly on the membrane and it will be better studied without mitochondrial receptors. We also need MFAs that lack interreceptor interactions, where the activities of fusogenic or docking proteins inserted in mitochondrial membranes can be specifically studied without intermitochondrial interactions. MFAs involving only mitochondrial membranes are essential to assess, in physiological-like conditions, how the fusion machinery is activated.

In a previous paper [28], we developed two MFAs to monitor the fusion of SUV with mitochondrial membranes and detected a membrane fusion component occurring at neutral pH. This process could be related to mitochondrial fusion, a complex membrane phenomenon that has not been studied at the molecular level. We have used fluorescence changes to detect in vitro membrane fusion events. Fluorescence MFAs assays are very sensitive, detecting a small number of successful fusion events, an important property for in vitro assays with dilute organelle suspensions. In addition, fluorescence-based MFAs are fast and they can be carried out as a test for fusogenic fractions collected during protein purification. This is important for our task of separating a complex mixture of OMM proteins into individual fusion-competent molecules and to obtain information about potential regulators. Proteins involved in fusion can have various roles; they can be part of a receptor that recognizes specific membranes before fusion, an activator–inhibitor regulatory protein molecule, or a protein subunit that belongs to a fusion-competent molecule.

Initially, using a very mild salt/detergent treatment that does not compromise mitochondrial inner membranes [28,32], we separated a soluble pool of OMM proteins (soluble fraction or SF) from mitochondria. SF increased MM and CM in a RSQ-based SUV/mitochondria MFA and the SF-stimulated RSQ was specifically sensitive to GTP (Fig. 1b). This experiment suggests that SF proteins may be involved in GTP-dependent membrane fusion occurring at neutral pH. In addition, this effect of guanine nucleotides required the presence of mitochondrial membranes; for the SUV/LUV mitochondria-free MFA, GTP lacked the effect present with the SUV/mitochondria MFA. The large low pH- and SF-dependent increase on MM and CM detected with the SUV/LUV MFAs suggests that SF enhances membrane contact between artificial membranes. Accordingly, experiments involving a very sensitive RET-based MM-MFA (Fig. 3) suggested that the presence in the OMM of some of the proteins solubilized in the SF pool is critical for intermitochondrial binding to occur. If mitochondria are washed at high ionic strength, almost no MM is detected. It should be noted that a purely collisional mechanism for the MM-mediated increase in RET will lead to an opposite prediction, i.e., that washing out proteins from the OMM makes available a greater membrane surface for membrane collisions that could produce intermembrane lipid transfer.

Membrane-mixing titrations showed unspecific fusion activity for G1 and a saturable, specific-like titration for G2 (Fig. 4b). This suggests that, even though G2 induces increased MM, only a limited number of G2-mediated contacts between SUV and mitochondria might occur. Results of the in vitro CM- and MM-MFAs were also differential for the G1 and G2 subfractions (Fig. 5). G1 increased CM and MM in all MFAs used, indicative of the presence of fusogenic proteins, while G2 had different effects between mitochondrial-containing and mitochondria-free MFAs. G2 has a more selective effect on the SUV-mitochondrial MFA, affecting predominantly MM rather than CM. Comparison between organelle-free MFAs (i.e., SUV/LUV) and mitochondrial MFAs reveals that the presence of mitochondria in these MFAs is necessary to produce fusion and the detected effect of GTP. Addition of GTP did not modify the SUV/LUV MFA with added G2, but GTP did increase G2-stimulated MM for the SUV/mitochondria MFA. The effects of G1 and G2 on MFAs are not additive when compared with SF (SF = G1 + G2), which may imply antagonistic G1–G2 activities or the formation of neutralizing complexes between components of the G1 and G2 subfractions. Addition of GTP had no effect on SF-stimulated changes for the SUV/LUV MFA and it only affected the SUV/mitochondria MFA. The stimulating activity of GTP on the mitochondrial MFAs (Figs. 1 and 5) indicates that complexes between proteins present in SF and the OMM could form in the presence of GTP, leading to complete mitochondrial fusion. As fusion occurs only when CM and MM are concurrent, the predominant MM effect of G2 suggests that the formation of a complex precedes an activating fusion step mediated by GTP.

Results from MFAs targeted to intermitochondrial fusion (Figs. 6–9) indicate that the differential properties of G1 and G2 extend to intermitochondrial fusion. Although both G1 and G2 increased MM between mitochondrial populations (Fig. 7), only G2's effect was sensitive to the addition of GTP. In the CM-mitochondria/mitochondria MFA (Fig. 9), G1 showed a concentration-dependent inhibition. The G1

subfraction thus increases contact between mitochondrial membranes, but it can block the ultimate fusion and mixing between the aqueous matrix spaces. A fusogenic protein contained in G1 might be binding to membranes in a non-productive fashion (e.g., wrong orientation with respect to the plane of the membrane), or the added excess of G1 can disrupt the normal fusion pathways by changing the stoichiometry of productive fusion complexes involving G1 and G2 proteins. Also, this result is inconsistent with a pure detergent-like effect of the G1 subfraction, as disruption of mitochondrial structure through unspecific fusion should release matrix-entrapped probes and lead to increased RET.

The positive effect of the G2 subfraction in both MM- and CM-mitochondria/mitochondria MFAs suggests that this subfraction contains proteins competent in membrane fusion. The fact that intermitochondrial MM and CM were both enhanced by GTP indicates that either a component of the G2 subfraction or its mitochondrial target leads to GTP-stimulated mitochondrial fusion. The effect of guanosine nucleotides suggests the participation of a GTP-dependent protein. While GDP and GDPβS lacked effect on stimulating intermitochondrial MM, GTP and its non-hydrolyzable analog GTPγS stimulated specifically the G2-dependent effect on MM (Fig. 7b). This effect of GTP and GTPγS is expected from the activity of a GTP-binding protein that is locked in its activated state by GTPγS, or displaced kinetically to such state by excess GTP. GDPβS could be inhibiting by competing with endogenous GTP [60], but this is a variable effect that requires up to mM-concentrations. Nonetheless, some trend towards inhibition was detected in the control and G1-treated sample.

A tabular summary (Table 1) of the experiments carried out with MFAs shows clear differential behavior between G1 and G2 added to the various MFAs systems progressively approaching mitochondrial fusion. G1 affects both MM and CM for the fusion between SUV and LUV or SUV and mitochondria (positive result for fusion). G2 induces limited stimulation of MM and CM for the SUV/LUV MFA and only affects MM in the SUV/mitochondria MFA. Only when mitochondria constitutes both interacting membranes (mitochondria/mitochondria MFAs), we detected significant G2-stimulated fusion with increased MM and CM. This will be consistent with a G2-dependent enhanced contact between mitochondrial membranes, probably involving the saturation of a limited number of OMM receptor sites (cf. Fig. 4b). Thus, G2-stimulated fusion, characterized by positive CM and MM, requires additional molecules (e.g., G1 components) in the opposing membranes.

As discussed before, addition of G1 and GTP gave similar results to the addition of G1 alone, but GTP did increase specifically the effects of G2. The preparation of G2 through selective GTP binding and the clear effect of GTP in G2-containing mitochondrial MFAs, both support the hypothesis that one of these proteins may be a member of the GTPase superfamily, specifically involved in the regulation of mitochondrial fusion. The use of data analysis tables such as Table 1 to codify the results from a set of MFAs leads to ready comparisons between subfractions obtained from protein separation and to clearly recognize the effects of potential activators (e.g., GTP). As the G2 subfraction is composed of only a few protein bands, isolation of these GTP-binding proteins is now feasible using the set of MFAs presented here. These MFAs have the additional advantage of incorporating both artificial membranes and reconstituted proteins, which will allow to assess the individual roles of lipid and protein components of biomembrane fusion.

Acknowledgements

The authors thank Dr. C. Nicchitta and Dr. T.J. McIntosh (Duke University) for valuable suggestions regarding the manuscript. We also thank Ian Heath and Gina DeFranco for technical assistance with organelle and protein preparations and Dr. E.R. Weiss (University of North Carolina at Chapel Hill) for the generous gift of guanosine nucleotides.

References

[1] A.B. Novikoff, The cell, in: J. Brachet, A.E. Mirsky (Eds.), Biochemistry, Physiology, Morphology, Vol. II, Academic Press, New York, 1961, pp. 299–421.
[2] C. Rouiller, Int. Rev. Cytol. Vol. IX, 1960, pp. 227–292.
[3] M. Tobioka, J.J. Biesele, J. Biophys. Biochem. Cytol. 2 (1956) 319–324, Suppl.

[4] S.G. Wildman, T. Hongladarom, S.I. Honda, Science 138 (1962) 434–436.
[5] D. Martz, R.J. Lasek, S.T. Brady, R.D. Allen, Cell Motil. 4 (1984) 89–101.
[6] I. Salmeen, P. Zacmanidis, G. Jesion, L.A. Feldkamp, Biophys. J. 48 (1985) 681–686.
[7] J. Bereiter-Hahn, M. Vöth, Microsc. Res. Tech. 27 (1994) 198–219.
[8] H. Otani, O. Tanaka, K. Kasai, T. Yoshioka, Anat. Rec. 222 (1988) 26–33.
[9] S.L. Young, E.K. Fram, C.L. Spain, E.W. Larson, Am. J. Physiol. 260 (1991) L113–L122.
[10] B. Tandler, C.L. Hoppel, Ann. New York Acad. Sci. 488 (1986) 65–81.
[11] J.-I. Hayashi, M. Takemitsu, Y. Goto, I. Nonaka, J. Cell Biol. 125 (1994) 43–50.
[12] D.B. Stern, D.M. Lonsdale, Nature 299 (1982) 698–702.
[13] P.E. Thorsness, T.D. Fox, Nature 346 (1990) 376–379.
[14] H. Takano, S. Kawano, T. Kuroiwa, Curr. Genet. 25 (1994) 252–257.
[15] H. Takano, S. Kawano, T. Kuroiwa, Curr. Genet. 26 (1994) 506–511.
[16] H.-P. Hoffmann, C.J. Avers, Science 181 (1973) 749–751.
[17] B.J. Stevens, J.G. White, Methods Enzymol. 56 (1979) 718–728.
[18] S.M. Burgess, M. Delannoy, R.E. Jensen, J. Cell. Biol. 126 (1994) 1375–1391.
[19] L.F. Sogo, M.P. Yaffe, J. Cell. Biol. 126 (1994) 1361–1373.
[20] S.A. Pratt, J. Morphol. 126 (1968) 31–66.
[21] R.S. Sohal, J.L. McCarthy, V.F. Allison, J. Ultrastruc. Res. 39 (1972) 484–495.
[22] K. Tanaka, T. Kanbe, T. Kuroiwa, J. Cell. Sci. 73 (1985) 207–220.
[23] K. Rohde, N.A. Watson, T. Cribb, Int. J. Parasitol. 21 (1991) 409–419.
[24] J. Nunnari, W.F. Marshall, A. Straight, A. Murray, J.W. Sedat, P. Walter, Molec. Biol. Cell 8 (1997) 1233–1242.
[25] A.A. Amchenkova, L.E. Bakeeva, Y.S. Chentsov, V.P. Skulachev, J. Cell. Biol. 107 (1988) 481–495.
[26] V.P. Skulachev, J. Membrane Biol. 114 (1990) 97–112.
[27] L.E. Bakeeva, Y.S. Chentsov, V.P. Schulachev, Biochim. Biophys. Acta 501 (1978) 349–369.
[28] J.D. Cortese, A.L. Voglino, C.R. Hackenbrock, J. Cell. Biol. 113 (1991) 1331–1340.
[29] J.D. Cortese, A.L. Voglino, C.R. Hackenbrock, Biochim. Biophys. Acta 1100 (1992) 189–197.
[30] J.D. Cortese, C.R. Hackenbrock, Biochim. Biophys. Acta 1142 (1993) 194–202.
[31] M. Zavortink, M.J. Welsh, J.R. McIntosh, Exp. Cell Res. 149 (1983) 375–385.
[32] C. Schnaitman, J.W. Greenwalt, J. Cell. Biol. 38 (1968) 158–175.
[33] J.D. Cortese, A.L. Voglino, C.R. Hackenbrock, Biochim. Biophys. Acta 1228 (1995) 216–228.
[34] G. Colombo, G.M. Carlson, H.A. Lardy, Biochemistry 17 (1978) 5321–5329.
[35] U.K. Laemmli, Nature 227 (1970) 680–685.
[36] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[37] I. Rustenbeck, W. Münster, S. Lenzen, Biochim. Biophys. Acta 1304 (1996) 129–138.
[38] W. Nieuwenhuizen, H. Kunze, G.H. De Haas, Methods Enzymol. 32 (1974) 147–154.
[39] J.D. Cortese, J.C. Vidal, P. Churchill, J.O. McIntyre, S. Fleischer, Biochemistry 16 (1982) 3899–3908.
[40] L.M. Geren, F. Millet, J. Biol. Chem. 256 (1981) 10485–10489.
[41] D. Hoekstra, T. de Boer, K. Klappe, J. Wilschut, Biochemistry 23 (1984) 5675–5681.
[42] D. Hoekstra, K. Klappe, T. de Boer, J. Wilschut, Biochemistry 24 (1985) 4739–4745.
[43] D. Hoekstra, N. Düzgünes, Methods Enzymol. 220 (1993) 15–32.
[44] A. Stutzin, FEBS Lett. 197 (1986) 274–280.
[45] N. Düzgünes, J. Wilschut, Methods Enzymol. 220 (1993) 3–14.
[46] H. Schneider, J.J. Lemasters, M. Höchli, C.R. Hackenbrock, J. Biol. Chem. 255 (1980) 3748–3756.
[47] P.S. Chen Jr., T.Y. Toribara, H. Warner, Anal. Chem. 28 (1956) 1756–1758.
[48] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, Biochim. Biophys. Acta 1061 (1991) 297–303.
[49] M.H. Davis, R.A. Altschuld, D.W. Jung, G.P. Brierley, Biochem. Biophys. Res. Commun. 149 (1987) 40–45.
[50] B.A. Scalettar, J.R. Abney, C.R. Hackenbrock, Proc. Natl. Acad. Sci. USA 88 (1991) 8057–8061.
[51] D.W. Jung, M.H. Davis, G.P. Brierley, Anal. Biochem. 178 (1989) 348–354.
[52] M. Poot, T.J. Kavanagh, H.C. Kang, R.P. Haugland, P.S. Rabinovitch, Cytometry 12 (1991) 184–187.
[53] R.P. Haugland, Handbook of Fluorescent Probes and Research Chemicals, 5th edn., Molecular Probes, Eugene, 1992.
[54] G.W. Kemble, T. Danielli, J.M. White, Cell 76 (1994) 383–391.
[55] J.L. Nieva, S. Nir, A. Muga, F.M. Goñi, J. Wilschut, Biochemistry 33 (1994) 3201–3209.
[56] N. Düzgünes, S.A. Shavnin, J. Membr. Biol. 128 (1992) 71–80.
[57] L. Zhang, H.P. Ghosh, J. Virol. 68 (1994) 2186–2193.
[58] W.E. Balch, K.R. Wagner, D.S. Keller, J. Cell. Biol. 104 (1987) 749–760.
[59] W. Almers, L.J. Breckenridge, A. Iwata, A.K. Lee, A.E. Spruce, F.W. Tse, Ann. New York Acad. Sci. 635 (1991) 318–327.
[60] G.G. Holz IV, S.G. Rane, K. Dunlap, Nature 319 (1986) 670–672.