Retargeting of the mitochondrial protein p32/gC1Qr to a cytoplasmic compartment and the cell surface

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SUMMARY

p32/gC1qR is a small acidic protein that has been reported to have a broad range of distinct functions and to associate with a wide array of cellular, viral and bacterial proteins. It has been found in each of the main cellular compartments including mitochondria, nucleus and cytoplasm and is also thought to be located at the plasma membrane and secreted into the extracellular matrix. The true physiological role(s) of p32 remains controversial because it has been difficult to reconcile all of the findings on protein interactions and the seemingly disparate observations on compartmentalisation. However, it has been proposed that p32 is somehow involved in transport processes connecting diverse cellular compartments and the cell surface. Here we show that native p32 appears to be localised mainly in the mitochondria and is not detectable on the cell surface. However, addition of a short tag to the N-terminus of p32 appears to block its mitochondrial targeting, resulting in redirection into a cytoplasmic vesicular pattern, overlapping with the endoplasmic reticulum. The redirection of p32 results in an alteration in and co-localisation with ER markers including calreticulin, a lumenal ER chaperone. Furthermore, we show both by immunofluorescence and cross-linking studies that this also results in cell-surface expression of p32. These results indicate that, at least under certain circumstances, p32 can be retargeted and may help to provide an explanation for the diverse observations on its localization.

Key words: p32, gC1qR, Mitochondria

INTRODUCTION

The human protein p32 has been identified and cloned by several groups using various different functional assays or two-hybrid screens. It was originally isolated through its co-purification with the nuclear splicing factor SF-2 (Krainer et al., 1991). Subsequently p32 was reported to inactivate SF-2 splicing activity and to associate with another splicing factor SRp30c, although in this latter case no effect on function was observed (Petersen-Mahrt et al., 1999). Several additional nuclear components have been reported to bind p32 including TFIIB, which led to the proposal that the protein may act as a bridging factor between TFIIB and transcriptional activators (Yu et al., 1995), and the lamin B receptor, from which it has been suggested that p32 may act as a link between the nuclear membrane and intranuclear substructures (Simos and Georgatos, 1994). Although additional nuclear proteins have been reported to bind p32, numerous observations of cytoplasmic binding factors have also been made. For example, in a yeast-two-hybrid screen, p32 was found to interact with the cytoplasmic domain of the α_{1b}-adrenergic receptor and appears to affect the cellular localisation and expression of the receptor (Xu et al., 1999). Furthermore, p32 was itself identified as a putative receptor protein for three extracellular plasma proteins namely kininogen, Factor XII, (both part of the coagulation and kinin-generating pathway) and the C1 complement component protein C1q (Ghebrehiwet et al., 1994; Herwald et al., 1996; Joseph et al., 1999a; Joseph et al., 1999b). With regard to this latter interaction p32 was found to bind to the globular heads of the C1q protein and was designated the gC1q receptor. Binding of p32 to C1q was shown to inhibit C1 mediated haemolysis, while enhancing chemotaxis (Ghebrehiwet et al., 1995; Ghebrehiwet et al., 1994; Leigh et al., 1998). Adding to the list of diverse binding partners, components of the extracellular matrix, hyaluronic acid and vitronectin**,** involved in cell-adhesion and motility, have also reported to bind to p32 (Deb and Datta, 1996; Lim et al., 1996). Finally, adding to the complexity concerning p32 localisation, fractionation and immunolocalisation studies showed that p32 was exclusively mitochondrial (Muta et al., 1997). Consistent with these conclusions, it was also reported that the yeast homologue of p32, Mam33, interacts with mitochondrial cytochrome b2 inter-membrane space sorting signal (Seytter et al., 1998).

In terms of interactions between p32 and components of infectious agents, a recent report showed convincingly that p32 could act as a receptor for invasion of the bacterium *Listeria monocytogenes* into mammalian cells (Braun et al., 2000). *Listeria* internalisation was mediated by a direct interaction of p32 with the bacterial surface protein InlB. Several viral proteins also appear to interact with p32. Direct binding of p32 with intracellular HIV Tat and EBV EBNA-1 was reported to augment transcriptional activation by the viral proteins (Wang et al., 1997; Yu et al., 1995). In addition to its effect on transcription, p32 has also been reported to stimulate HIV RNA export by the viral Rev protein (Luo et al., 1994). In another study the adenovirus structural core protein V (a viral genome associated protein) was shown to bind p32, an

interaction that was suggested to mediate aspects of adenovirus intracellular transport (Matthews and Russell, 1998). The herpesvirus Orf P protein has also been found to bind to p32 in yeast two-hybrid analyses, although in this latter case no functional connections were established (Bruni and Roizman, 1996).

Although the physiological relevance of the observations of binding to this diverse array of proteins remains to be established, the observations on compartmentalisation and trafficking of p32 appear particularly difficult to reconcile. Although recent cell fractionation studies of human macrophage cells and yeast cells indicate that endogenous p32 is almost exclusively localised in the mitochondrial matrix (Muta et al., 1997; Seytter et al., 1998), results from immunofluorescence studies remain equivocal. Several reports confirm p32 localisation to the mitochondria (Dedio et al., 1998; Matthews and Russell, 1998; Muta et al., 1997), whereas the same or different studies also detect p32 in the nucleus (Matthews and Russell, 1998; Petersen-Mahrt et al., 1999) and on the cell surface of neutrophils, mast cells, platelets and fibroblasts (Eggleton et al., 1995; Ghebrehiwet et al., 1995; Gupta et al., 1991; Peerschke et al., 1994). Secreted p32 has been identified from cultured cells and in human sera (Peterson et al., 1997; van den Berg et al., 1995).

Because of the diverse nature of some of the interacting proteins and localisation of p32, both intracellular and extracellular, it has been proposed that p32 could be a component of trafficking pathways connecting the nucleus, cytoplasm and mitochondria and the export pathway to the cell surface. But it has also been suggested that reports concerning function outside the mitochondria need to be treated with caution. Here we report results that may help reconcile p32 localisation in different studies and show that blocking the N-terminal mitochondrial-targeting signal of p32 causes it to be diverted away from the mitochondria to a globular cytoplasmic pattern partly co-localising with ER components. This relocalised p32 co-localised with ER components such as calreticulin, inducing a change in calreticulin localisation from a normal reticular pattern to a pronounced punctate/vesicular pattern. No significant changes were observed in localisation of Golgi markers. The redirected p32 could also be detected on the cell surface. The results indicate that, at least under certain circumstances, p32 can be targeted or diverted to different compartments allowing the possibility that such retargeting could happen as part of normal p32 processing, for example in different cell types. However, the results also provide a possible explanation for the diverse observations made by different groups, for example using different versions or tagged forms of the protein. They also indicate that general caution needs to exercised particularly with regard to addition of tags for detection, which may fundamentally affect functional properties of the protein under study.

MATERIALS AND METHODS

Cells and transfection

COS-1 cells were maintained in Dulbecco's modified minimal essential medium containing 10% newborn calf serum. Cells were transfected with plasmid DNA (typically 4 μg/10^6 cells) using the calcium phosphate precipitation method with BES (*N*,*N*-bis(2-hydroxyl)-2-aminoethanesulfonic acid)-buffered saline, as previously described (Greaves and O'Hare, 1989). Cells were processed for western blotting or immunofluorescence approximately 40 hours after transfection.

Plasmids

The plasmid YW59 (Wang et al., 1997) containing p32 tagged at its N-terminus with a Flag tag was a kind gift from D. Hayward. To construct a version of p32 tagged only at its C-terminus, p32 sequences were amplified from pYW59 using a p32 specific forward primer which contained a KpnI site; 5′, CCGGTACCATGCTGCCT-CTGCTGCGCTGCTG, 3, and a reverse primer containing a XbaI site; 5′, CCTCTAGACTCTGGCTCTTGACAAAACTCTTGAGG, 3. The PCR product was digested with KpnI and XbaI and ligated into pcDNA 3.1mychisB (Invitrogen) similarly digested with KpnI and XbaI. This resulted in the construction of an expression vector for full length p32, containing its native N-terminus and tagged at its C-terminus with the ten-residue myc epitope tag (Fig. 1b, p32-myc). A construct containing p32 double-tagged at both its N-terminus and C-terminus was made by PCR amplification from pYW59 using the forward primer; 5′, CCGGTACCATGGACTACAAGGACGATG, 3′ to include the FLAG epitope tag sequence together with the same reverse primer as for p32-myc (Fig. 1b, flag-p32-myc). The recombinant constructs were sequenced to confirm the reading frame and tags.

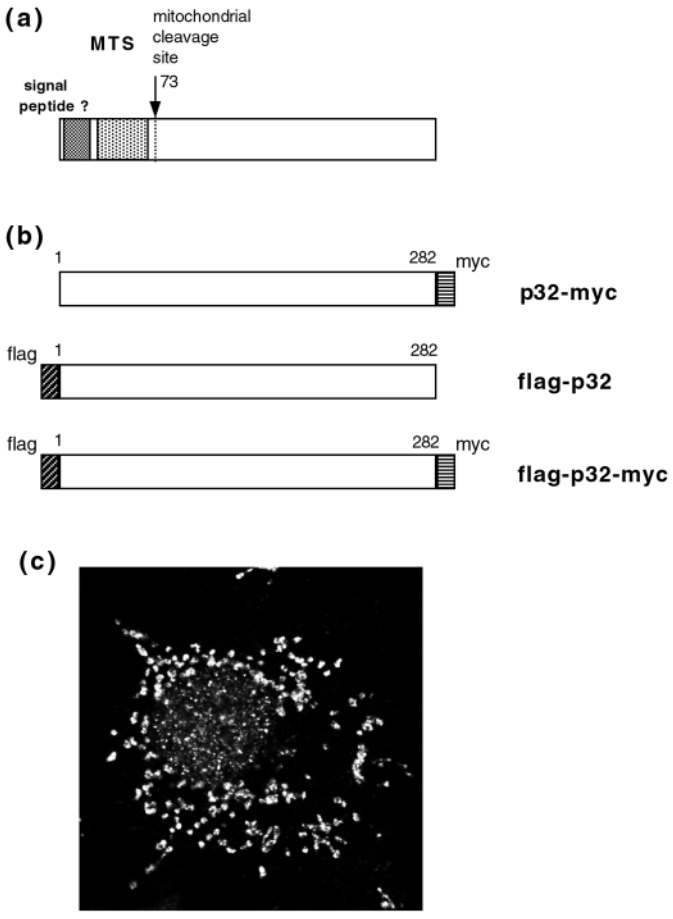

Fig. 1. (a) Schematic representation of p32, including various predicted motifs. (b) Summary of p32 constructs p32-myc, flag-p32 and flag-p32-myc. The boxes indicate the epitope tags. (c) Typical staining pattern of the endogenous p32 in COS-1 cells observed with an anti-p32 polyclonal antibody.

Immunofluorescence

COS cells seeded on glass coverslips were transfected with the appropriate expression vector and approximately 40 hours later, were washed twice with PBS and fixed for 20 minutes at –20°C with 100% methanol. The cells were then blocked in PBS containing 10% calf serum for 10 minutes at room temperature. Primary antibodies were added in the same solution and incubated for 45 minutes at room temperature. Following two 5 minute washes with PBS, secondary antibodies were added in blocking buffer and incubated for 15 minutes. After an additional two washes in PBS the coverslips were mounted in Mowiol (Sigma) containing 2.5% 1,4-diazabicyclo-2.2.2-octane to reduce bleaching. For analysis of cell surface location of p32, cells were fixed in 4% paraformaldehyde in PBS at room temperature for 10 minutes and processed without permeabilisation. Antibodies used in this study and their dilutions were as follows; monoclonal antibody to the myc-epitope (R950-25, Invitrogen, 1:200), monoclonal antibodies to γ-Adaptin (A4325, 1:10) and Flag-M2 (F3165, 1:200) were obtained from Sigma; polyclonal anti-Flag antibody (Sc-807, 1:20) was obtained from Santa Cruz; polyclonal anti-calreticulin (NB600101, 1:20) was from Novus Biologicals; polyclonal anti-Prohibitin (RB-292, 1:20) from Labvision; polyclonal anti-β-COP (PA1-061, 1:200) was from Affinity Bioreagents. The polyclonal rabbit antiserum against p32 was kindly provided by D. Matthews. Secondary antibodies were fluorescein isothiocyanate conjugated anti-mouse (F1-1000, 1:100) or anti-rabbit immunoglobulin (F1-2000, 1:100) both from Vector Laboratories and tetramethylrhodamine B isothiocyanate conjugated anti-mouse (T7782, 1:200) or anti-rabbit immunoglobulin (T5268, 1:200) both obtained from Sigma. Localisation was examined by confocal microscopy using a Zeiss LSM410 confocal microscope and images annotated using Adobe Photoshop software.

Cell-surface biotinylation

After transfection, COS cells were washed twice with ice-cold PBS [pH 8.0], drained and incubated at room temperature for 30 minutes in 2 ml of PBS [pH=8.0] containing 0.5 mg/ml sulfosuccinimidyl-6-biotinamido-hexanoate (Sulfo-NHS-LC-biotin, Pierce). The cells were then washed twice in PBS, once in PBS containing 10 mM glycine, once in PBS containing 0.1% BSA and finally twice in PBS in order to remove and quench any remaining biotinylation reagent. Cells were then suspended in 250 μl lysis buffer (50 mM Tris pH=8.0, 500 mM NaCl, 0.1% sodium-dodecyl-sulphate, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.02% sodium azide, 0.5 mM PMSP and Complete™ protease inhibitor cocktail (Boehringer Mannheim). After 20 minutes incubation at 0°C, the cell suspension was centrifuged at 12000 rpm for 2 minutes at 4°C. The supernatant was transferred to a fresh tube containing 50 μl of 50% slurry of Streptavidin agarose beads (Pierce) in lysis buffer and the samples incubated for 1 hour at 4°C with rotation. The beads were then washed five times in 300 μl lysis buffer and bound proteins solubilised in 50 μl of 2% SDS gel loading buffer. Samples were subject to electrophoresis on SDS-polyacrylamide gels and p32 species analyzed by western blot analysis using either anti-myc or anti-flag antibody.

RESULTS

The reports of p32 in many different intracellular and extracellular compartments prompted us to examine and compare by immunofluorescence the location of endogenous p32 and exogenous epitope-tagged p32 expressed from a series of plasmids containing different tagged versions of the protein (Fig. 1b). The antibodies used were a polyclonal antibody previously raised against the endogenous protein (Matthews and Russell, 1998) and well-defined monoclonal antibodies to the different tags. In agreement with previous observations using the polyclonal antibody (Matthews and Russell, 1998), we detected endogenous p32 in a punctate cytoplasmic pattern, frequently including thin thread-like structures, typical of a mitochondrial localisation (Fig. 1c; see also Fig. 3a and Fig. 4a). Also as previously reported, weaker but distinct nuclear diffuse staining of p32 was observed with this antibody. A virtually identical cytoplasmic pattern was observed for the transfected C-terminal epitope tagged p32 protein using the anti-myc monoclonal antibody (Fig. 2a; Fig. 3a, p32-myc) suggesting that this reflects the endogenous p32 population. Dual staining of the transfected cells using antibody to the mitochondrial protein, prohibitin (Ikonen et al., 1995), together with the anti-myc antibody demonstrated virtually complete overlap, confirming the mitochondrial location of p32-myc (Fig. 3a-c). Note detailed inspection at higher magnifications and resolution indicated that the p32-myc/prohibitin co-localisation was not absolutely precise, consistent with the

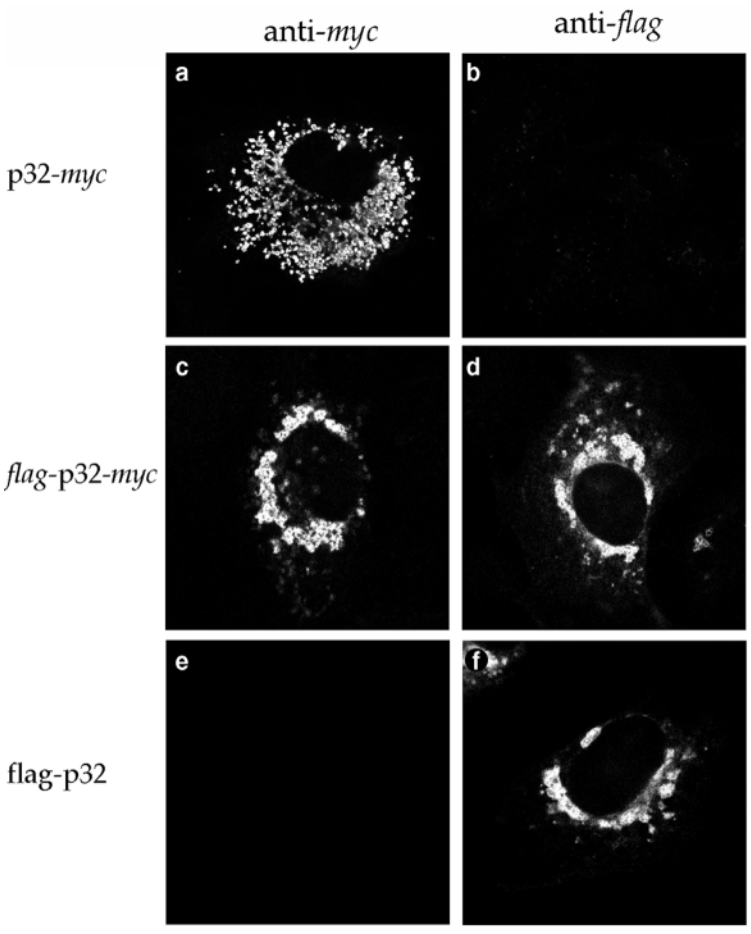

Fig. 2. N-terminal epitope tagging of p32 changes its distribution. Indirect immunofluorescence of COS-1 cells transfected with C-terminal-tagged p32-myc (a,b), double-tagged flag-p32-myc (c,d) and N-terminal-tagged flag-p32. (e,f). Cells were fixed 40 hours after transfection and incubated with antibodies against the c-myc epitope (a,c,e) or against the Flag epitope. Cells were analysed by confocal microscopy and typical images illustrated.

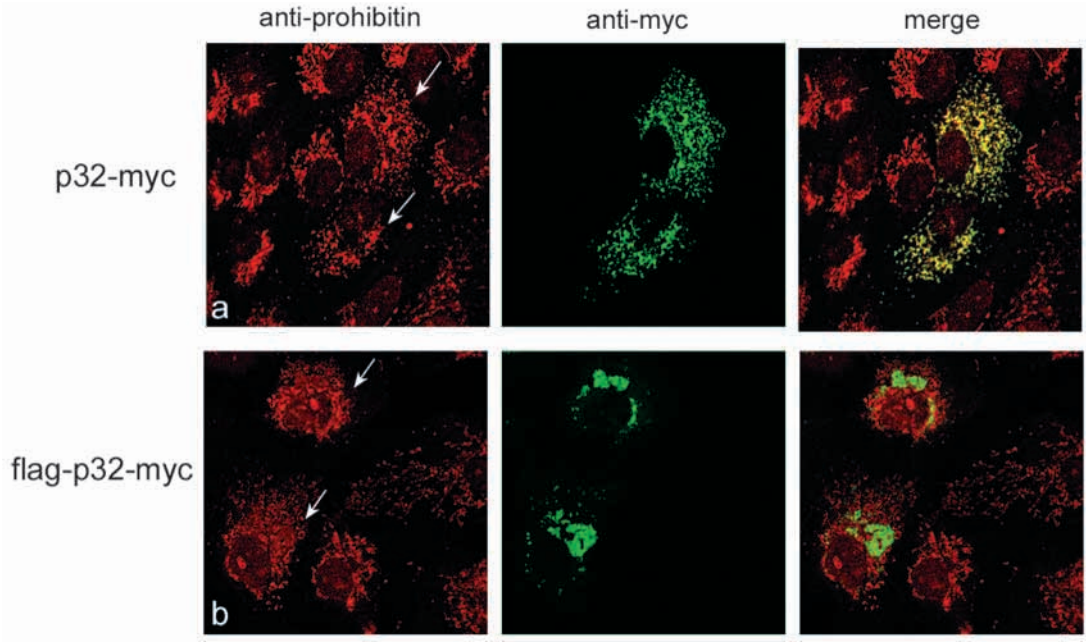

Fig. 3. P32-myc localisation in mitochondria is blocked by N-terminal residues. Cells were transfected with p32-myc (panel a) and flag-p32-myc (panel b) and stained with anti-prohibitin (red) and anti-myc antibodies (green). The individual images are shown merged in the right-hand panel. Arrows indicate cells transfected expressing the p32 species. p32-myc and prohibitin showed almost complete co-localisation, appearing yellow in the merged image, whereas the flag-p32 appears in a distinct globular pattern showing little overlap with the mitochondria.

known localisation of prohibitin in the inner membrane of mitochondria (Ikonen et al., 1995) and the previously reported localisation of p32 to the mitochondrial matrix (Muta et al., 1997). Furthermore, consistent with the mitochondrial location, computer analysis of the p32 sequence indicates that it contains a mitochondrial targeting signal at its N-terminus (Fig. 1a; see also Fig. 5).

In contrast to the concordance of the cytoplasmic patterns, p32-myc did not exhibit any detectable nuclear accumulation (Fig. 2a; Fig. 3a; Fig. 4a). This suggests that the p32 polyclonal antibody may cross-react with a nuclear protein, although evidence against this possibility includes observation of the same nuclear pattern using affinity-purified serum, and the finding that purified p32 blocked detection (Matthews and Russell, 1998). However, alternative explanations for the failure of p32-myc to localise in the nucleus, for example that the myc epitope was selectively masked in any nuclear p32, or that the exogenous p32 was specifically not transported to the nucleus, although possible, are less likely in our view.

Surprisingly, both of the versions of p32 that contained an N-terminal tag, flag-p32 or flag-p32-myc, showed a distinctly different pattern of localisation, typical examples of which are shown in Fig. 2c-f. Instead of the discrete small punctate and threadlike cytoplasmic pattern observed for p32-myc, the N-terminally tagged versions were found in relatively large globules, frequently concentrated around the nucleus. (Note that the patterns shown for flag-p32-myc illustrate different fields for the flag and myc antibodies). It was also clear from the dual staining experiments that in contrast to p32-myc, neither of the N-terminally tagged proteins co-localised with mitochondrial prohibitin (Fig. 3b). Identical results, showing mitochondrial co-localisation of p32-myc but altered localisation to a globular pattern for the N-terminally tagged versions were obtained in additional cell types including Veros, BHK and HeLas.

To examine the possibility that the addition of the N-terminal tag was simply inducing malfolding and aggregation of the protein, we examined aggresome formation in cells expressing flag-p32. Aggresome formation has been proposed to be indicative of protein malfolding, resulting in formation of a condensed perinuclear accumulation of the disrupted protein surrounded by collapsed intermediate filaments (Johnston, 1998). We found none of the hallmarks of aggresome formation, nor any effect on or co-localisation with a number of other markers including ubiquitin or heat shock proteins (data not shown). Furthermore the flag-p32 was expressed normally and not subject to any increased proteolysis or altered breakdown products (Fig. 5).

We conclude from these observations, together with the results below, that the addition of an N-terminal tag does not simply induce p32 malfolding and aggregation. Rather, we propose that the addition of the N-terminal ten residues of the tag either blocks localisation to mitochondria and/or redirects it to another cell location. Because the ten amino acid Flag-tag does not contain any known signal sequences itself, we believe that interference with normal recognition of the mitochondrial signaling is the most likely explanation for our results.

Cytoplasmic location of re-targeted p32

To examine whether the altered location of the N-terminally tagged proteins represented retargeting to any particular compartment we therefore examined co-localisation with a number of cellular ER and Golgi markers (Fig. 4). In each of the fields shown, short arrowheads indicate untransfected cells not expressing the transfected p32 proteins, whereas the long arrows illustrate expressing cells.

We first examined co-localisation of the flag-tagged p32 proteins with the ER marker calreticulin, a Ca^{2+}- and carbohydrate-binding protein, which is localised to the ER lumen (Fig. 4b). Unexpectedly, the results showed a complex relationship between the expression of flag-p32 and calreticulin localisation. In cells expressing the flag-p32 (Fig. 4b, long arrows), we observed an altered calreticulin distribution, from the reticular lattice pattern normally observed (Fig. 4a,b, short arrowheads) to a condensed globular

pattern. The altered calreticulin pattern showed some but only partial overlap with the flag-p32 pattern (Fig. 4b, merged, top cell) and in many cells relatively little overlap was evident with the p32 accumulating adjacent to the calreticulin-rich structures (Fig. 4b, merged bottom cell). But in addition, in a population of cells, the flag-p32 was seen to exhibit very pronounced co-localisation with the altered calreticulin pattern (Fig. 4c). Virtually identical results were obtained for flag-p32 co-localisation with calnexin, an ER-membrane-bound chaperone similar to calreticulin (Fig. 4d). Note that in cells expressing the normal mitochondrial targeted p32-myc, no co-localisation with and no effect on the calreticulin distribution was observed (Fig. 4a, compare expressing cells, long arrowheads, with adjacent non-expressing cells, short arrowheads).

In contrast to the results with calreticulin, when examining distribution of in relation to the Golgi marker γ-adaptin, no co-localisation with flag-p32 was observed, nor was there any detectable effect, in numerous fields examined, of the flag-p32 on γ-adaptin distribution in any expressing cells (Fig. 4f). We also examined localisation of the flag-p32 in relation to that of β-COP, a component of intermediate compartment cargo vesicles (Fig. 4e). In this case we found two types of patterns. In many cells we observed little overlap, with the β-COP in a distinctive Golgi-like perinuclear pattern, quite separate from the globular flag-p32 (Fig. 4e, long arrow, top cell). The pattern of β-COP in these cells was indistinguishable from that normally seen (compare with short arrowheads). However in a significant number of cells, rather than the clearly

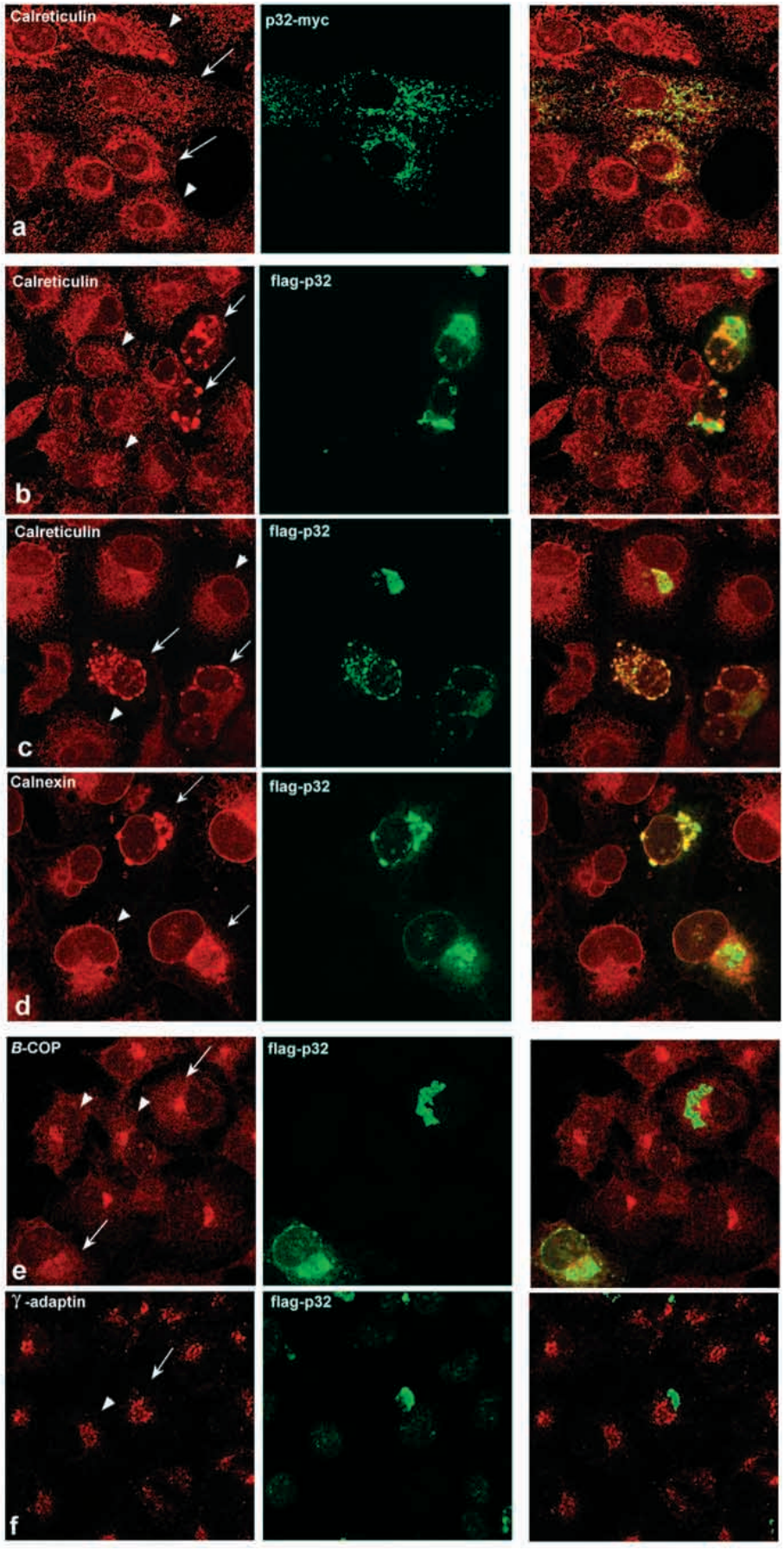

Fig. 4. Localisation of flag-p32 compared to a number of ER-Golgi components. Staining with antibodies against markers for endogenous calreticulin, calnexin, γ-adaptin, and β-COP are in red. Flag-p32 is shown in green. In each of the panels showing individual cellular components, cells expressing the p32 species are indicated by long arrows, whereas for comparison cells not expressing the p32 are shown by short arrowheads. Two separate fields (panels b,c) are illustrated for calreticulin relationship to flag-p32, whereas panel a shows the calreticulin pattern for the normally localised p32-myc. The individual images are shown merged in the right-hand panel.

separate locations, the β-COP and flag-p32 exhibited overlap, particularly in the area closest to the nuclear membrane (Fig. 4e, long arrow, bottom cell). It appeared that in these latter cells, the β-COP pattern was somewhat more disperse than in the expressing cells where no overlap was observed.

Clearly, the flag-p32 is not being retargeted to an unaltered cellular compartment in a simple fashion. Perhaps one interpretation consistent with the results with the ER resident protein calreticulin (and partly for β-COP) is that the flag-p32 is redirected to the ER, but that such redirection has an effect on at least certain ER components (while having little effect on the Golgi). We note that the extreme N-terminal 19 residues encompass a hydrophobic region, which is identified from prediction algorithms to be a potential signal peptide (Fig. 1a).

However, whatever the precise mechanism and location, the results clearly show that the flag-p32 does not target normally, and the redirection away from the mitochondria appears to affect the ER. This is in striking contrast to the p32-myc, which targets mitochondria normally.

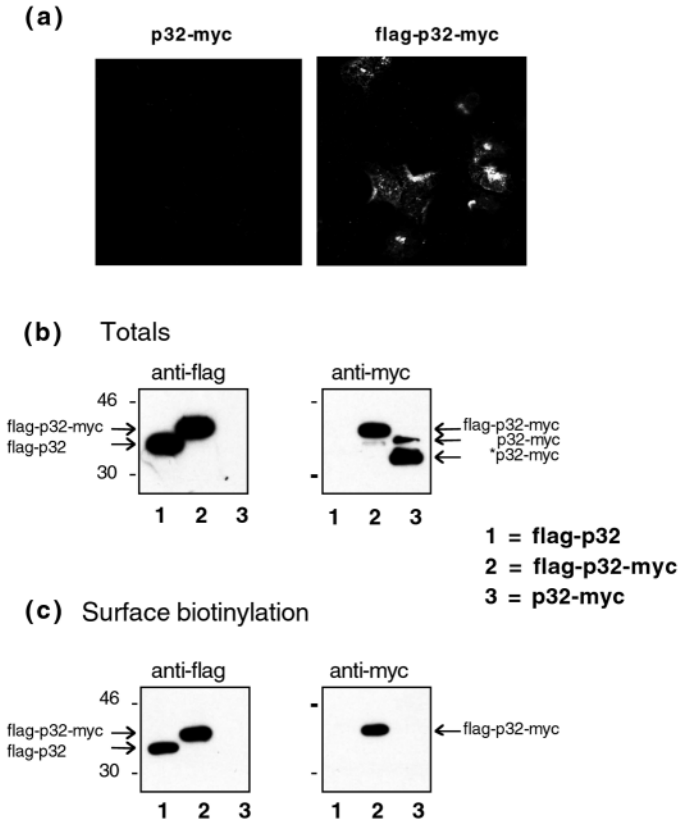

Fig. 5. Cell-surface expression of flag-p32 species. (a) Staining of non-permeabilised cells demonstrates cell-surface expression of flag-p32 constructs. COS-1 cells transfected with p32-myc or flag-p32-myc were fixed in 4% paraformaldehyde and analysed with anti-myc antibody. (b,c) Cell-surface biotinylation of p32 expression constructs. COS-1 cells were transfected with flag-p32 (lanes 1), flag-p32-myc (lanes 2) and p32-myc (lanes 3) and 40 hours later labelled with membrane-impermeable Sulfo-NHS-LC-biotin. (b) Total extracts (~5×10^4 cells per lane). (c) Streptavidin-bound, cell-surface expressed, extract (~5×10^5 cells per lane). Proteins were detected by western blot using the indicated antibodies. * indicates proteolytically cleaved p32.

Retargeted p32 proteins appear on the cell surface

One of the earliest identified activities of p32 was that as a receptor for the globular heads of complement component and p32 was shown to be expressed on the cell surface (Ghebrehiwet et al., 1994). Further work indicated that p32 was a receptor for several extracellular plasma proteins (Ghebrehiwet et al., 1994; Herwald et al., 1996; Joseph et al., 1999a; Joseph et al., 1999b) and that p32 bound components of the extracellular matrix (Deb and Datta, 1996; Lim et al., 1996). These earlier observations, together with our results on the retargeted p32, prompted us to test whether the different p32 versions could be detected on the cell surface. Cells transfected as before with each of the vectors were fixed in 4% paraformaldehyde and processed for p32 localisation without permeabilisation (Fig. 5). No cell-surface expression could be detected with the polyclonal antibody detecting endogenous p32 (data not shown). Likewise, the C-terminally tagged p32-myc protein, which exhibited the typical mitochondrial localisation, could not be detected on the cell surface (Fig. 5a). Surprisingly, in parallel assays, the N-terminally tagged flag-p32-myc could be readily detected on the extracellular surface using the same antibody and identical conditions (Fig. 5a). Likewise, the flag-p32-myc protein (and the flag-p32) could also be detected on the surface with the anti-Flag antibody, suggesting that both termini were exposed on the cell surface (data not shown). Although the surface pattern of the flag-p32-myc (or flag-p32) did not resemble that of a diffusible membrane protein, the results were reproducibly obtained and were consistent with biochemical surface labelling (see below).

Cell-surface biotinylation of N-terminally tagged p32 proteins

Expression of proteins on the cell surface can be also be detected biochemically, using the membrane-impermeable *N*-hydroxy succinimide-ester of biotin, Sulfo-NHS-LC-biotin, to covalently biotinylate available extracellular proteins of the plasma membrane (Altin and Pagler, 1995). COS-1 cells were transfected with each of the p32 expression vectors, flag-p32, flag-p32-myc or p32-myc and 24 hours later the monolayers were washed and treated with Sulfo-NHS-LC-biotin followed by inactivation and removal of the biotinylation agent. Extracts were then made and a portion retained for analysis of the total expression levels of the p32 species, while specifically biotinylated proteins were isolated using streptavadin coated agarose beads. These biotinylated cell-surface proteins were then assayed by western blot for the presence of epitope-tagged p32 proteins. Expression levels of the flag-p32 and flag-p32-myc constructs were virtually identical as detected by the anti-flag antibody (Fig. 5b, anti-flag, lanes 1 and 2), as were levels of the flag-p32-myc and p32-myc, as detected by the anti-myc antibody (Fig. 5b, anti-myc, lanes 2 and 3). The pairwise comparisons of expression levels indicate that as expected each of these constructs was expressed at virtually identical levels.

Consistent with the results from the immunofluorescence studies, both of the N-terminally tagged p32 proteins were detected by surface biotinylation (Fig. 5c, anti-flag, lanes 1 and 2; anti-myc, lane 2). The C-terminally tagged p32-myc protein, lacking the N-terminal tag, could not be detected by surface biotinylation again consistent with the immunofluorescence results (Fig. 5c, lane 3). Note that the biotinylated flag-p32-myc protein could be detected with both antibodies, thus

revealing identically migrating proteins (Fig. 5c, compare lanes 2), which suggests that the surface-located protein had not been proteolytically cleaved. Despite the expression of p32-myc in virtually identical amounts to flag-p32-myc, this species was not detected on the cell surface. Finally, we note that only the p32-myc protein was detectably cleaved, as the western blot of the total cell extract revealed a doublet, with the majority of the protein migrating slightly faster than the upper, lower-abundance band (Fig. 5b, anti-myc, lane 3). Because these bands were detected with the C-terminal antibody, the results are entirely consistent with the proposal that p32–myc is processed by cleavage at the N-terminus, reflecting cleavage of the mitochondrial signalling sequence. Furthermore, as indicated above and consistent with the observation of retargeting by immunofluorescence, little if any processing or cleavage was detected for either of the N-terminally tagged proteins.

DISCUSSION

The human protein p32/gC1qR has been reported to have a broad range of functions, reflecting observed associations with a diverse array of cellular, viral and bacterial components. It has been found in each of the main cellular compartments including the mitochondria, nucleus and cytoplasm, and has also been observed on the plasma membrane and secreted into the extracellular matrix. It is difficult to reconcile all of the findings on protein interactions and the seemingly disparate observations on compartmentalisation. For example, on the one hand the protein has been proposed to be a surface component acting as an internalisation factor for infection by Listeria, whereas on the other it has been proposed to be an internal chaperone for trafficking adenovirus to the nucleus. Clearly, understanding these activities of p32 would have important consequences for understanding the replicative life cycles of these pathogens, but the true physiological role(s) of p32 remains controversial. We wished to examine p32 localisation by immunofluorescence and surface labelling in an attempt to help reconcile such disparate findings. We found that exogenously supplied p32, containing a well-defined epitope tag at its C-terminus, appears to be exclusively mitochondrial. We did not detect the protein in the nucleus or on the cell surface, at least in the cell types under study, and even though comparatively overexpressed, we found little or no protein in cytoplasmic compartments outside the mitochondria. These observations are consistent with those of Muta and colleagues who found p32 to be present exclusively in mitochondria (Muta et al., 1997). However, we also found that p32 could be redirected. Addition of an epitope tag to the N-terminus of p32 appears to block the mitochondrial targeting sequence in the protein, resulting in redirection to the cytoplasm, in a globular/perinuclear pattern, in association with an altered ER. Furthermore, from both immunofluorescence and cross-linking studies we found that this retargeting also results in the appearance of p32 on the cell surface. (Note that since the flag-p32 was found on the cell surface, it is possible that endosomal retrieval contributes to its internal localisation).

The interference by the N-terminal ten amino acid tag on the mitochondrial targeting sequence (MTS) of p32 could be due to the net negative charge (–3) in the tag, or by a spacing alteration within the N-terminus. From the results examining calnexin and calreticulin, it appears that expression of the redirected flag-p32 results in a pronounced effect on the distribution of at least some ER resident proteins. In many cells expressing flag-p32 there was an alteration in calreticulin (and calnexin) from a typical ER lattice pattern to a globular/vecisular pattern with which the flag-p32 showed minor co-localisation. In a subpopulation of cells the altered calreticulin and flag-p32 showed distinct co-localisation. There was little effect on and no co-localisation with the Golgi component γ-adaptin, whereas for β-COP there was some overlap in a population of cells.

We believe that the redirection of p32 and the effect on the resident ER proteins are linked and propose the following explanation. Thus, although mitochondrial presequences are frequently cleaved upon entry to the matrix (see below), the targeting signals are complex and frequently dual in nature, so that, for example with an additional signal, after the cleaved matrix targeting signal, proteins are inserted into the inner mitochondrial membrane in either orientation (Neupert, 1997). The dual signals may be separable or interdigitated. An explanation consistent with the all the results is that the tag actually blocks the MTS part of its N-terminus, but that additional signals in p32 target it into the ER. Because the N-terminal tag is still present (by IF and by sizing), the protein could be in the lumen of the ER, or if a membrane signal is present (which should normally operate in mitochondria), the flag-p32 may be inserted into the ER membrane. As a consequence, whether disposed lumenally or on the outside of the ER, this could result in direct interaction and alteration of the ER proteins or, for example, ER stress. Alternatively, the effect of the redirected p32 could be due to the possibility, based on structural determination, of it acting as a pore protein for small molecules (Jiang, 1999). It has also been suggested, based on the p32 structure and similarity with calsequestrin, a Ca^{2+} storage protein, that it could be involved in the regulation of Ca^{2+} levels. The effect on calreticulin may even be more specific, as both p32 and calreticulin have been reported to bind a common component (C1q) and to interact with each other directly (Ghebrehiwet et al., 1997).

Whatever the precise mechanism, the results on the relocalisation and cell-surface detection are consistent with the proposal that the residues within the N-terminus of p32 retains some sort of targeting function, but that the MTS aspect normally dominates. It may not, however, be the case that any putative N-terminal signal can be readily separated form the MTS. For example, with the A-kinase anchoring protein (DAKAP-1), a splice variant gains an additional N-terminal 33 residues, switching the location of the protein from mitochondria to ER (Huang et al., 1999). But the extra residues do not contain an independent ER targeting signal and instead it appears that a part of the mitochondrial targeting sequence functions in concert with the extra residues to form an ER signalling motif. Note that in one report, when the complete 73 N-terminal residues were cleaved from p32, it was not specifically compartmentalised, but was found in a diffuse location in the cytoplasm and nucleus (Muta et al., 1997). Further work should help address this point.

Other examples of dual targeting include monooxygenase P450 isoforms, which contain a chimeric ER and mitochondrial signal sequence at the N-terminus, and where

the ER targeting signal appears to compete with the MTS (Anandatheerthavarada et al., 1999; Bhagwat et al., 1999). The mitochondrial or ER destination can be modulated by phosphorylation at a distant protein kinase A site, where phosphorylation activates the signal for mitochondrial transport. Interestingly, surface-bound and serum-released p32 are phosphorylated by protein kinase C (Rao et al., 1997). Two out of three predicted protein kinase C sites in p32 lie within the p32-MTS and phosphorylation could possibly neutralise the positive charges important in MSF binding to target signals (Komiya et al., 1994). Site-directed mutagenesis of these potential phosphorylation sites on p32 could reveal whether such a mechanism really takes place. Alternative splicing of the p32 N-terminus does not seem a conceivable mechanism for p32 targeting because close examination of the genomic 5′ region and exon 1 of p32 did not reveal any alternative high-probability splice donor or acceptor sites.

Proteins targeted to the ER-Golgi that lack specific retention signals are transported to the plasma membrane. Classically, proteins that enter the secretory pathway would have their N-terminal signal sequences cleaved from them, yet we detect the cell surface p32 with the N- and the C-terminal probes, indicating that the full-length protein is present on the surface. However, there are many precedents for protein export in the absence of cleavable signal sequences and by non-classical routes (Cleves et al., 1996; Rubartelli et al., 1990; Rubartelli et al., 1992). Although the mechanism by which the flag-p32 appears on the surface is not clear, its export could involve indirect associations with the ligands or extracellular matrix components such as vitronectin and hyaluronic acid, with which it interacts. Retention on the surface (we have not detected p32 in the medium) is likely to be via indirect contacts, possibly to the same cell-surface components, rather than via membrane insertion.

It seems reasonable to propose that at least some of the diverse observations on trafficking of p32 to locations outside the mitochondria have physiological relevance, yet this remains controversial. Our observations with the C-terminally tagged protein are consistent with the proposal that p32 is normally exclusively a mitochondrial protein, and that previous demonstrations of promiscuous associations and locations are artifactual. Such artifacts could have resulted from immunofluorescence analysis of different tagged versions of the protein (e.g. Wang et al., 1997) and, in biochemical analysis, from non-specific electrostatic interactions (the pI of p32 is 4.2) directly or even indirectly, e.g via RNA binding, with a variety of target proteins. The results demonstrate that caution needs to be exercised generally when studying protein compartmentalisation, particularly with regard to the addition of tags for detection, which may affect trafficking pathways of the protein under study. Nevertheless, the possibility that blocking the p32 mitochondrial targeting by an N-terminal tag reflects a cellular regulatory mechanism warrants further study and the results allow the prospect that such retargeting could occur as part of p32 processing, helping to account for diverse observations on its localisation.

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REFERENCES

Altin, J. G. and Pagler, E. B. (1995). A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. *Anal. Biochem.* **224**, 382-389.

Anandatheerthavarada, H. K., Biswas, G., Mullick, J., Sepuri, N. B., Otvos, L., Pain, D. and Avadhani, N. G. (1999). Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at ser128. *EMBO J.* **18**, 5494-5504.

Bhagwat, S. V., Biswas, G., Anandatheerthavarada, H. K., Addya, S., Pandak, W. and Avadhani, N. G. (1999). Dual targeting property of the N-terminal signal sequence of P4501A1. Targeting of heterologous proteins to endoplasmic reticulum and mitochondria. *J. Biol. Chem.* **274**, 24014-24022.

Braun, L., Ghebrehiwet, B. and Cossart, P. (2000). gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of Listeria monocytogenes. *EMBO J.* **19**, 1458-1466.

Bruni, R. and Roizman, B. (1996). Open reading frame P–a herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mRNA. *Proc. Natl. Acad. Sci. USA* **93**, 10423-10427.

Cleves, A. E., Cooper, D. N., Barondes, S. H. and Kelly, R. B. (1996). A new pathway for protein export in Saccharomyces cerevisiae. *J. Cell Biol.* **133**, 1017-1026.

Deb, T. B. and Datta, K. (1996). Molecular cloning of human fibroblast hyaluronic acid-binding protein confirms its identity with P-32, a protein co-purified with splicing factor SF2. Hyaluronic acid-binding protein as P-32 protein, co- purified with splicing factor SF2. *J. Biol. Chem.* **271**, 2206-2212.

Dedio, J., Jahnen-Dechent, W., Bachmann, M. and Muller-Esterl, W. (1998). The multiligand-binding protein gC1qR, putative C1q receptor, is a mitochondrial protein. *J. Immunol.* **160**, 3534-3542.

Eggleton, P., Ghebrehiwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B. and Tauber, A. I. (1995). Identification of a gC1q-binding protein (gC1q-R) on the surface of human neutrophils. Subcellular localization and binding properties in comparison with the cC1q-R. *J. Clin. Invest.* **95**, 1569-1578.

Ghebrehiwet, B., Kew, R. R., Gruber, B. L., Marchese, M. J., Peerschke, E. I. and Reid, K. B. (1995). Murine mast cells express two types of C1q receptors that are involved in the induction of chemotaxis and chemokinesis. *J. Immunol.* **155**, 2614-2619.

Ghebrehiwet, B., Lim, B. L., Peerschke, E. I., Willis, A. C. and Reid, K. B. (1994). Isolation, cDNA cloning, and overexpression of a 33-kD cell surface glycoprotein that binds to the globular "heads" of C1q. *J. Exp. Med.* **179**, 1809-1821.

Ghebrehiwet, B., Lu, P. D., Zhang, W., Keilbaugh, S. A., Leigh, L. E., Eggleton, P., Reid, K. B. and Peerschke, E. I. (1997). Evidence that the two C1q binding membrane proteins, gC1q-R and cC1q-R, asociate to form a complex. *J. Immunol.* **159**, 1429-1436.

Greaves, R. and O'Hare, P. (1989). Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. *J. Virol.* **63**, 1641-1650.

Gupta, S., Batchu, R. B. and Datta, K. (1991). Purification, partial characterization of rat kidney hyaluronic acid binding protein and its localization on the cell surface. *Eur. J. Cell Biol.* **56**, 58-67.

Herwald, H., Dedio, J., Kellner, R., Loos, M. and Muller-Esterl, W. (1996). Isolation and characterization of the kininogen-binding protein p33 from endothelial cells. Identity with the gC1q receptor. *J. Biol. Chem.* **271**, 13040-13047.

Huang, L. J., Wang, L., Ma, Y., Durick, K., Perkins, G., Deerinck, T. J., Ellisman, M. H. and Taylor, S. S. (1999). NH2-Terminal targeting motifs direct dual specificity A-kinase- anchoring protein 1 (D-AKAP1) to either mitochondria or endoplasmic reticulum. *J. Cell Biol.* **145**, 951-959.

Ikonen, E., Fiedler, K., Parton, R. G. and Simons, K. (1995). Prohibitin, an antiproliferative protein, is localized to mitochondria. *FEBS Lett.* **358**, 273-277.

Jiang, J., Zhang, Y., Krainer, A. R. and Xu, R. M. (1999). Crystal structure of human p32, a doughnut-shaped acidic mitochondrial matrix protein. *Proc. Natl. Acad. Sci. USA* **96**, 3572-3577.

Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998). Aggresomes: a cellular response to misfolded proteins. *J. Cell Biol.* **143**, 1883-1898.

Joseph, K., Ghebrehiwet, B. and Kaplan, A. P. (1999a). Cytokeratin 1 and

gC1qR mediate high molecular weight kininogen binding to endothelial cells. *Clin. Immunol.* **92**, 246-255.

Joseph, K., Ghebrehiwet, B., Peerschke, E. I., Reid, K. B. and Kaplan, A. P. (1996). Identification of the zinc-dependent endothelial cell binding protein for high molecular weight kininogen and factor XII: identity with the receptor that binds to the globular "heads" of C1q (gC1q-R). *Proc. Natl. Acad. Sci. USA* **93**, 8552-8557.

Joseph, K., Shibayama, Y., Nakazawa, Y., Peerschke, E. I., Ghebrehiwet, B. and Kaplan, A. P. (1999b). Interaction of factor XII and high molecular weight kininogen with cytokeratin 1 and gC1qR of vascular endothelial cells and with aggregated Abeta protein of Alzheimer's disease. *Immunopharmacology* **43**, 203-210.

Komiya, T., Hachiya, N., Sakaguchi, M., Omura, T. and Mihara, K. (1994). Recognition of mitochondria-targeting signals by a cytosolic import stimulation factor, MSF. *J. Biol. Chem.* **269**, 30893-30897.

Krainer, A. R., Mayeda, A., Kozak, D. and Binns, G. (1991). Functional expression of cloned human splicing factor SF2: homology to RNA-binding proteins, U1 70K, and Drosophila splicing regulators. *Cell* **66**, 383-394.

Leigh, L. E., Ghebrehiwet, B., Perera, T. P., Bird, I. N., Strong, P., Kishore, U., Reid, K. B. and Eggleton, P. (1998). C1q-mediated chemotaxis by human neutrophils: involvement of gClqR and G-protein signalling mechanisms. *Biochem. J.* **330**, 247-254.

Lim, B. L., Reid, K. B., Ghebrehiwet, B., Peerschke, E. I., Leigh, L. A. and Preissner, K. T. (1996). The binding protein for globular heads of complement C1q, gC1qR. Functional expression and characterization as a novel vitronectin binding factor. *J. Biol. Chem.* **271**, 26739-26744.

Luo, Y., Yu, H. and Peterlin, B. M. (1994). Cellular protein modulates effects of human immunodeficiency virus type 1 Rev. *J. Virol.* **68**, 3850-3856.

Matthews, D. A. and Russell, W. C. (1998). Adenovirus core protein V interacts with p32–a protein which is associated with both the mitochondria and the nucleus. *J. Gen. Virol.* **79**, 1677-1685.

Muta, T., Kang, D., Kitajima, S., Fujiwara, T. and Hamasaki, N. (1997). p32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phosphorylation. *J. Biol. Chem.* **272**, 24363-24370.

Neupert, W. (1997). Protein import into mitochondria. *Ann. Rev. Biochem.* **66**, 863-917.

Peerschke, E. I., Reid, K. B. and Ghebrehiwet, B. (1994). Identification of a novel 33-kDa C1q-binding site on human blood platelets. *J. Immunol.* **152**, 5896-5901.

Petersen-Mahrt, S. K., Estmer, C., Ohrmalm, C., Matthews, D. A., Russell, W. C. and Akusjarvi, G. (1999). The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation. *EMBO J.* **18**, 1014-1024.

Peterson, K. L., Zhang, W., Lu, P. D., Keilbaugh, S. A., Peerschke, E. I. and Ghebrehiwet, B. (1997). The C1q-binding cell membrane proteins cC1q-R and gC1q-R are released from activated cells: subcellular distribution and immunochemical characterization. *Clin. Immunol. Immunopathol.* **84**, 17-26.

Rao, C. M., Deb, T. B., Gupta, S. and Datta, K. (1997). Regulation of cellular phosphorylation of hyaluronan binding protein and its role in the formation of second messenger. *Biochim. Biophys. Acta* **1336**, 387-393.

Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. (1992). Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J. Biol. Biochem.* **267**, 24161-24164.

Rubartelli, A., Cozzolino, F., Talio, M. and Sitia, R. (1990). A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J.* **9**, 1503-1510.

Seytter, T., Lottspeich, F., Neupert, W. and Schwarz, E. (1998). Mam33p, an oligomeric, acidic protein in the mitochondrial matrix of Saccharomyces cerevisiae is related to the human complement receptor gC1q-R. *Yeast* **14**, 303-310.

Simos, G. and Georgatos, S. D. (1994). The lamin B receptor-associated protein p34 shares sequence homology and antigenic determinants with the splicing factor 2-associated protein p32. *FEBS Lett.* **346**, 225-228.

van den Berg, R. H., Faber-Krol, M., van Es, L. A. and Daha, M. R. (1995). Regulation of the function of the first component of complement by human C1q receptor. *Eur. J. Immunol.* **25**, 2206-2210.

Wang, Y., Finan, J. E., Middeldorp, J. M. and Hayward, S. D. (1997). P32/TAP, a cellular protein that interacts with EBNA-1 of Epstein-Barr virus. *Virology* **236**, 18-29.

Xu, Z., Hirasawa, A., Shinoura, H. and Tsujimoto, G. (1999). Interaction of the alpha(1B)-adrenergic receptor with gC1q-R, a multifunctional protein. *J. Biol. Chem.* **274**, 21149-21154.

Yu, L., Zhang, Z., Loewenstein, P. M., Desai, K., Tang, Q., Mao, D., Symington, J. S. and Green, M. (1995). Molecular cloning and characterization of a cellular protein that interacts with the human immunodeficiency virus type 1 Tat transactivator and encodes a strong transcriptional activation domain. *J. Virol.* **69**, 3007-3016.