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Small GTP-Binding Proteins in the Nuclei of Human Placenta

Phoebe Phillips,[1] and Murray Thomson,[2]*

[1]University of Western Sydney Nepean, PO Box 10, Kingswood, NSW, 2747, Australia
[2]School of Biological Sciences, University of Sydney, NSW, 2006, Australia

Abstract Mitochondria and crude nuclei containing fractions from human placenta have been shown to contain proteins which bind [α^{32}P]-GTP. Prior to this study the number of GTP-binding proteins in placental nuclei and their nucleotide specificity was not known. Also unknown was the identity of any of the GTP-binding proteins in mitochondria of human placenta. Nuclei and mitochondria were purified from human placental extracts by sedimentation. Proteins were separated by electrophoresis and transferred to nitrocellulose membranes. Overlay blot with [α^{32}P]-GTP identified two nuclei proteins with approximate molecular weights of 24 and 27 kDa. Binding of [α^{32}P]-GTP to the 27 and 24 kDa proteins was significantly displaced by guanine nucleotides but not by adenine, thymine or cytosine nucleotides or deoxy (d) GTP. Western blot with a specific antibody to Ran identified a band at 27 kDa in nuclei and in mitochondrial fractions. These data indicate that both nuclei and mitochondria contain 24 and 27 kDa GTP-binding proteins. The GTP-binding proteins in nuclei display binding specificity for guanine nucleotides and the hydroxylated carbon 2 on the ribose ring of GTP appears essential for binding. It will be important in future studies to determine the functions of these small GTP-binding proteins in the development and physiology of the placenta. J. Cell. Biochem. 84: 100–107, 2002.

Key words: nuclei; GTP-binding proteins; placenta; Ran; mitochondria

GTP-binding proteins are involved in the regulation of a variety of physiological processes in many cell and tissue types. GTP-binding proteins are activated when bound to GTP and inactivated when inherent GTPase activity converts the bound GTP to GDP. GTP-binding proteins are often divided into groups on the basis of their size and quaternary structure. The heterotrimeric GTP-binding protein group are often referred to as G proteins and are composed of a GTP-binding α subunit, as well as, β and γ subunits [Bourne, 1994]. The molecular weight of a heterotrimeric G protein α subunit usually resides within a range of 35–50 kDa [Bourne, 1994, 1997]. The heterotrimeric G proteins are often involved in the transmission of a cellular signal through the plasma membrane [Bourne, 1994]. Additionally, there is the family of monomeric GTP-binding proteins which often have a molecular weight of 14–33 kDa and due to this size have been named small GTP-binding proteins [Takai et al., 2001]. Small GTP-binding proteins have been found to be involved in processes including gene expression, cytoskeletal organization [Takai et al., 2001] and membrane fusion [Thomson, 1998].

Recently, overlay blot with [α^{32}P]-GTP has identified several GTP-binding proteins in the human placenta and it has been proposed that these may be important regulators of placental physiology [Thomson et al., 1998]. The placenta is an ephemeral organ that supplies the fetus with nutrients and the mother and the fetus with hormones [Petraglia et al., 1996]. Because the healthy development and function of the placenta is crucial to the health of both fetus and mother it would appear important to characterise the GTP-binding proteins present in this organ. GTP-binding proteins of molecular weights 24, 27, 33, 37 and 52 kDa have been

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Phoebe Phillips's present address is Faculty of Medicine, University of New South Wales, NSW, 2052, Australia.

*Correspondence to: Murray Thomson, School of Biological Sciences (F07), The University of Sydney, NSW, 2006, Australia. E-mail: mthomson@bio.usyd.edu.au

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shown to be localised in the mitochondria of human placenta [Thomson et al., 1998]. Whether these proteins were similar or identical to known GTP-binding proteins found in other parts of the cell was not known at this time [Thomson et al., 1998]. GTP-binding proteins of 24 and 27 kDa were found to be present in crude cell debris fractions containing nuclei, but it was not determined whether these were present in purified preparations of nuclei [Thomson et al., 1998].

In many species including *Xenopus* [Gorlich et al., 1994], mouse [Coutavas et al., 1994] and rat [Clarkson et al., 1996] the participation of the GTP-binding protein Ran is needed for protein transport into the nucleus. While Ran has been identified in the nuclei of many tissues and cells, there have only been a few studies to determine whether there are small GTP-binding proteins other than Ran in the nucleus and these have focused on the rat. Overlay blot with [α^{32}P]-GTP has shown the existence of 25 and 26 kDa GTP-binding proteins in rat neuronal nuclei [Bhullar et al., 1999], while in rat liver nuclei, 23, 24.5, 25 and 26 kDa proteins have been located [Rubins et al., 1990]. Prior to this study Ran or other small GTP-binding proteins had not been identified in the nuclei of human placental tissue.

In the present study we determined whether there were [α^{32}P]-GTP binding proteins in nuclei from human placenta and whether these were specific GTP-binding proteins. Additionally, we used specific antibodies to determine whether three known GTP-binding proteins (Ran, Rac and Rho) with molecular weights in the 20–28 kDa range were located in mitochondria or nuclei from human placenta.

METHODS

Isolation of Mitochondria From Placenta

Human term placentae was obtained from Westmead Hospital, Australia and placed in chilled phosphate buffered saline (PBS). The maternal umbilical vein and fetal arteries were perfused with PBS to remove blood. The preparation of mitochondria was carried out using a modification of the method by Adams et al. [1989]. Pieces of placental tissue approximately 1 cm^3 were homogenised in buffer A (Tris–HCl 20 mM, mannitol 210 mM, sucrose 70 mM) using a motor driven Teflon pestle. Next, the homogenate was centrifuged at 700*g* for 10 min at 4°C in a Sorvall Ultra Pro 80 ultracentrifuge (Fixed angle rotor and 35 ml tubes). The resulting supernatant was kept at 4°C and the pellet was re-extracted twice in buffer A. The supernatants were combined and centrifuged at 9,750*g* for 15 min at 4°C. The resulting mitochondrial pellet was collected and resuspended in 200 ml of buffer A. This suspension was centrifuged three times at 9,750*g* at 4°C. Following this the supernatant was removed and the mitochondrial pellets resuspended in buffer A.

Isolation of Nuclei From Placenta

The isolation of human placental nuclei was carried out as described by Boivin and Beliveau [1995]. For the preparation of nuclei, 2 g of homogenised placental tissue prepared as above was re-homogenised in 20 ml of 2.4 M sucrose, 3.3 mM $CaCl_2$ and 0.04% trasylol. The homogenate was then filtered through three layers of cheesecloth and centrifuged at 40,000*g* for 60 min. The supernatant was removed and the pellet was resuspended in 2 ml of 1 M sucrose, 1 mM $CaCl_2$ and 0.04% trasylol. The suspension was centrifuged three times at 3,000*g* for 5 min. The final nuclear pellet was resuspended in 250 mM sucrose, 1 mM $CaCl_2$ and 0.04% trasylol, and stored at −70°C.

Assay of Protein Concentration

The concentration of each mitochondrial sample was determined via the Pierce BCA Protein Assay as per the manufacturer's instructions. The samples were read at 562 nm on the Biorad microplate reader (Biorad Model 550 microplate reader).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) was performed using the Biorad Mini Protean II system. The separation of mitochondrial and nuclear proteins involved resuspending the samples in SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS w/v, 10% glycerol and 5% β-mercaptoethanol). The samples were then heated at 95°C for 3–5 min and then centrifuged for 5 min at 3,000 rpm. Samples were loaded onto 12% SDS gels that contained a 4% stacking gel and run at 100 V for approximately 1.5 h. Protein bands obtained for mitochondrial and nuclei samples were detected using coomassie stain and silver

stain (Biorad Silver Stain Plus Kit) as per the manufacturer's instructions.

Overlay Blot Detection of GTP-Binding Proteins

GTP overlay assays were performed on isolated nuclei. The samples were first run on 12 % SDS gels and then transferred electrophoretically to nitrocellulose. The lanes on the nitrocellulose were marked using ponceau stain. The stain was removed with TBS (20 mM Tris and 0.9% NaCl, pH 7.5). The nitrocellulose was then blocked with 5% skim milk in TBS for 20 min. The method described by Zeuzem et al. [1992] was followed to perform the assay in order to characterise GTP binding in proteins from the nuclei. The nitrocellulose membrane was washed twice in incubating buffer (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.3% Tween 20, 1 mM K_2HPO_4/KH_2PO_4) for 10 min. The membrane was left to incubate for 1 h in 1 μl of [α^{32}P]-GTP (3×10^{-6}mol/L) per 10 ml of buffer. The membrane was then washed five times for 10 min in incubating buffer. The membrane was allowed to dry and then wrapped in plastic food wrap and exposed to X-ray film at −70°C.

To investigate the nuclear fractions for specificity for guanine nucleotides, membranes containing SDS–PAGE separated proteins were pre-incubated for 20 min with a 10 × concentration of the unlabelled nucleotides AMP, ADP, ATP, GMP, GDP, GTP, CTP, ITP, TTP, and dGTP. On completion, [α^{32}P]-GTP was added to the membranes (in the presence of unlabelled nucleotides) for 1 h. The membranes were then washed as outlined above in incubating buffer and exposed to X-ray film at −70°C.

Immunodetection of Ran in the Nucleus

The immunoreactivity of Ran was detected by Western blot using the purified goat polyclonal antibody Ran (C-20) obtained from Santa Cruz Biotechnology (USA). Nuclei containing 50 μg of protein were separated by 12% SDS–PAGE and transferred by electrophoresis to nitrocellulose using a Biorad Mini Trans-Blot system. The membranes were blocked in 5% skim milk in TBS with 0.05% Tween-20 (TTBS) for 1 h. The membranes were incubated for 1 h at room temperature with polyclonal anti-Ran/TC4 antiserum (1 μg/ml) in 5% skim milk in TTBS. The membranes were then washed three times for 5 min each with TTBS and incubated with the secondary antibody (gamma anti-goat coupled to horseradish peroxidase obtained from Santa Cruz Biotechnology; 1:2,000) for 45 min. The membrane was then rinsed with TTBS (3 × 5 min) and finally with TBS. The membrane was then incubated in chemiluminescence luminol reagent (ECL Du Pont) and exposed to X-ray film.

In addition, a Western blot was performed with polyclonal antibodies for Rac and Rho (obtained from Santa Cruz Biotechnology) using the same procedure as above.

Analysis of SDS–PAGE Results

The molecular weight of proteins and band intensity was estimated with the aid of the scientific program NIH IMAGE.

Statistical Analysis

One way analysis of variance (ANOVA) was used to analyse the data. $P < 0.05$ was considered to be significant.

RESULTS

SDS–PAGE of Nuclear Extracts

Proteins expressed in the nuclei and mitochondria from human placentae were analysed using one-dimensional SDS–PAGE and detected using coomassie blue (Fig. 1). The samples from nuclei and mitochondria showed banding patterns, which spanned the 200–20 kDa molecular weight range. Staining the gels with silver did not result in additional bands becoming apparent (results not shown).

GTP Overlay of Nuclei Fractions

GTP overlay assay was used to determine whether GTP-binding proteins were detectable in nuclei isolated from human placenta. Various protein amounts were analysed by this method and a dose dependant identification of 24 and 27 kDa GTP-binding proteins was observed (Fig. 2). A 3- and 18-h exposure of the membrane with labelled proteins is shown in Figure 2a,b, respectively. The 24 and 27 kDa proteins from a 50 μg loading and a 2- and 6-h autoradiograph exposure is shown in Figure 2c,d.

Specificity of GTP-Binding Proteins

Overlay blot analysis was performed in the presence and absence of unlabeled nucleotides to determine the specificity of the affinity of the 24 and 27 kDa proteins for GTP. Nitrocellulose

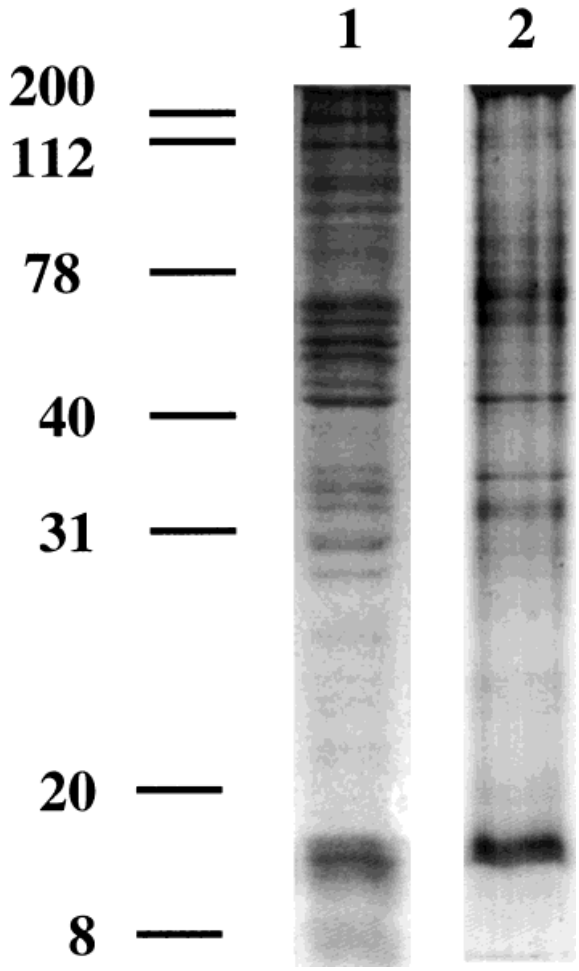

Fig. 1. SDS–PAGE of proteins from human placenta. Fifty micrograms of protein were added to each lane. **Lane 1** shows nuclear proteins, **lane 2** shows mitochondrial proteins. The gel was stained using coomassie blue.

bound proteins were incubated with the radiolabel and 10 × concentration of the unlabeled nucleotides AMP, ADP, ATP, GMP, GDP, GTP, CTP, ITP, and TTP. Results in Figure 4 show that AMP, ADP, ATP, CTP, ITP, and TTP did not significantly inhibit the binding of [α^{32}P]-GTP. GMP, however, significantly ($P < 0.05$) displaced the [α^{32}P]-GTP from the 24 and 27 kDa proteins (Fig. 3). Furthermore, both GDP and GTP completely abolished the binding of labelled GTP ($P < 0.001$) (Fig. 3).

Participation of Ribose Hydroxyl Group in GTP Binding

To determine whether the oxygen atom in the hydroxyl group attached to carbon 2 in GTP is necessary for binding of [α^{32}P]-GTP to 24 and 27 kDa, nuclei proteins immobilised on nitrocellulose were preincubated with radio-labeled [α^{32}P]-GTP with and without a 10 × concentration of deoxy (d) GTP. There was no significant ($P = 0.06$) displacement of [α^{32}P]-GTP in the presence of dGTP (Fig. 3).

Rac, Rho and Ran Immunoreactivity

In order to obtain the identity of the small GTP-binding proteins expressed within placental nuclei, Western blots were performed using polyclonal antibodies for two GTP-binding proteins Ran and Rac which have molecular weights comparable to the two GTP-binding proteins in the nuclei of the placenta. Results revealed the presence of Ran immunoreactivity in nuclei obtained from the placenta (Fig. 4). GTP-overlay performed on a membrane that had been analysed for Ran antibody binding showed that Ran immunoreactivity was present at precisely the same position as the 27 kDa [α^{32}P]-GTP binding protein (Fig. 4). In contrast, Rac and Rho were found to be absent from the nuclei in all three placentas tested (results not shown).

The presence of ran was also investigated and found to be present in percoll purified mitochondria of human placentae (Fig. 5). The concentration of Ran did not significantly ($P = 0.12$) differ between the mitochondria and the nucleus.

DISCUSSION

In a previous study primarily on mitochondria, crude fractions from human placenta which contained nuclei were shown to contain two small molecular weight GTP-binding proteins [Thomson et al., 1998]. In the present study nuclei were separated from crude fractions and the GTP-binding proteins in the nuclei fractions were analysed for their protein content. As shown in Figure 1, nuclei and mitochondria fractions stained nonspecifically for protein showed a wide range of protein bands over the 200–20 kDa range. For both nuclei and mitochondria at loadings of 50 μg, all protein bands in the gel were sharply defined and no bands exhibited vertical streaking, a symptom of overloaded gels. This indicated that 50 mg of protein was an appropriate amount to be loaded onto the gel.

Overlay blot with [α^{32}P]-GTP showed major bands of 24 and 27 kDa (Fig. 2) and binding displayed a dose responsive manner (Fig. 2).

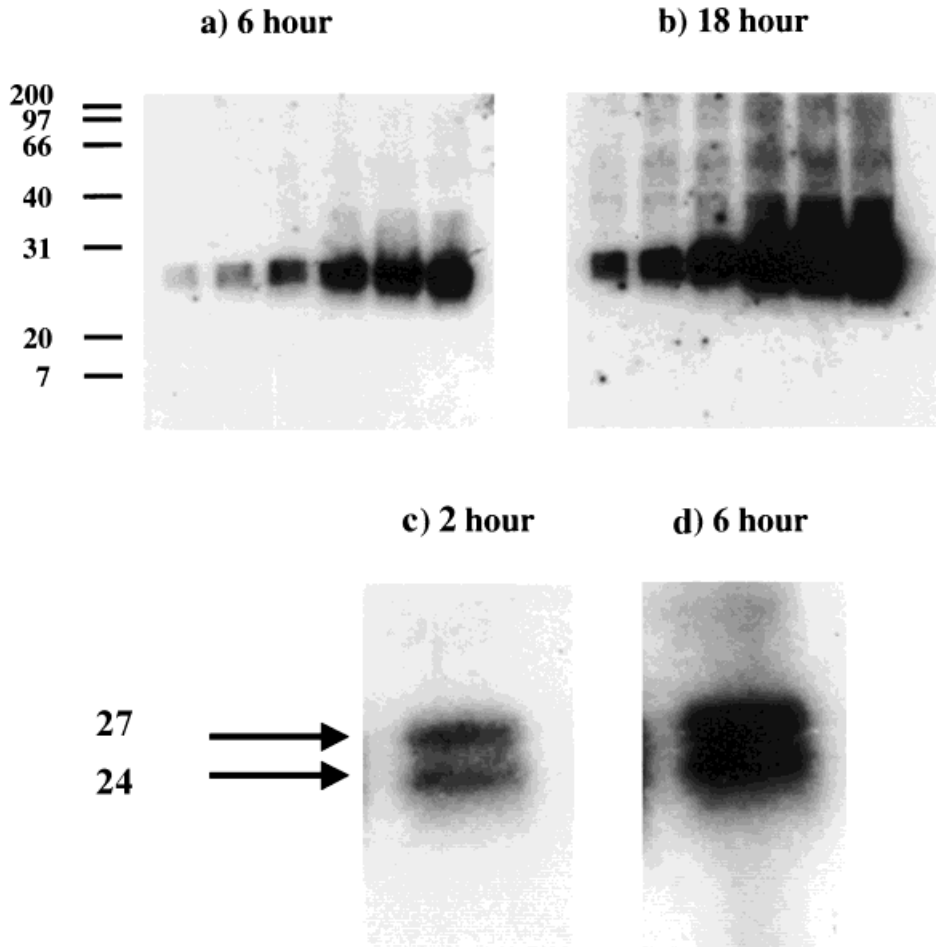

Fig. 2. Binding of [α^{32}P]-GTP to placental nuclei proteins. In (**a**) and (**b**) **Lanes 1**, **2**, **3**, **4**, **5** and **6** contain 5, 10, 20, 50, 75 and 100 μg of protein respectively. Figures a and b show exposure times of 6 and 18 h respectively. The effect of time of exposure on detection of the 24 and 27 kDa [α^{32}P]-GTP binding proteins is shown in (**c**) and (**d**) which show 2 and 6 h autoradiograph exposure times, respectively. The experiment was repeated a total of three times using different placentae with similar results.

This pattern is similar to the results reported for mitochondria [Thomson et al., 1998] and a 50 μg loading was in the linear range of response. Higher doses with an autoradiograph exposure time of 18 h showed some GTP-binding activity in the 30–70 kDa range although signal to noise ratio in this region was low and discrete bands were not apparent. Figure 1 demonstrates that there are a number of discrete protein bands in the 30–200 kDa range, however, the [α^{32}P]-GTP binding properties of the proteins in this molecular weight range are absent or negligible. It would appear therefore that the 24 and 27 kDa GTP-binding proteins in the nuclei are in the small GTP-binding protein class and appear likely to be monomeric [Bourne, 1997; Thomson, 1998].

Because the 24 and 27 kDa proteins are close in molecular weight and the overlay blot technique creates a 'halo' effect around the bands, discrimination of the 24 and 27 kDa bands from each other is sometimes problematic [Thomson et al., 1998]. The exposure time of the overlay blot in autoradiography can also effect resolution between the 24 and 27 kDa bands as shown in Figure 2.

GTP-binding proteins have affinity for both GDP and GTP and much lower affinity for nonguanosine nucleotides [Denslow et al., 1991]. The specificity of GTP-binding by the 24 and 27 kDa proteins in placental nuclei was examined by pre-incubating blots with 10 × the concentration of unlabelled nucleotide (Fig. 3). The pyrimidine nucleotides tested (CTP and TTP) did not significantly effect the binding of [α^{32}P]-GTP to 24 and 27 kDa proteins. It appears therefore that a purine is essential for a nucleotide to interact with the binding site of

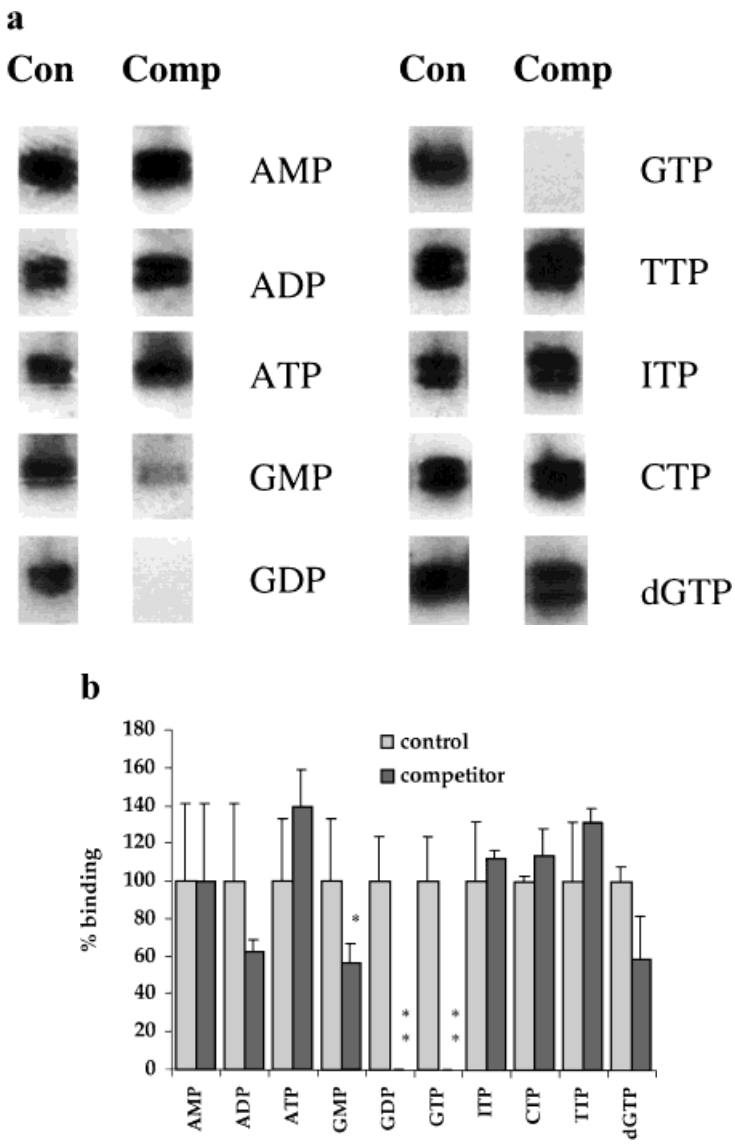

Fig. 3. Specificity of [α^{32}P]-GTP binding to the 24 and 27 kDa proteins. Equal amounts (50 μg protein) of nuclear extract from three different placentae were subjected to overlay blot in the presence of a 10-fold excess of unlabelled nucleotide (competitor) or saline vehicle only (control). The values are the means ± SD. (**a**) Shows the autoradiograph of the proteins while (**b**) shows densitometry of the bands.

24 and 27 kDa GTP-binding proteins. The adenosine nucleotides also did not show any significant displacement of [α^{32}P]-GTP. This indicated that a) the amino group in the purine must be at carbon 2 rather than carbon 6 and/or b) an oxygen atom double bonded to carbon 6 is required for binding to 24 and 27 kDa proteins. In addition, ITP had no significant effect on the binding of [α^{32}P]-GTP. ITP resembles GTP except for the absence of the amino group at position 2, thus the amino group at carbon 2 must by itself be important in GTP binding. In contrast, both GDP and GTP completely abolished the binding of [α^{32}P]-GTP. GMP lacks the β-phosphate group, and the low affinity for GMP displayed by the 24 and 27 kDa proteins indicates that the β-phosphate contributes to the binding interaction [Denslow et al., 1991].

In order to investigate the importance of the ribose ring in binding of [α^{32}P]-GTP to GTP-binding proteins, deoxy-GTP was used as a competitor (Fig. 3). The results showed that dGTP had no significant effect on the binding of [α^{32}P]-GTP to 24 and 27 kDa nuclear proteins. This indicates that the 2′-hydroxyl group present on GTP is needed for binding.

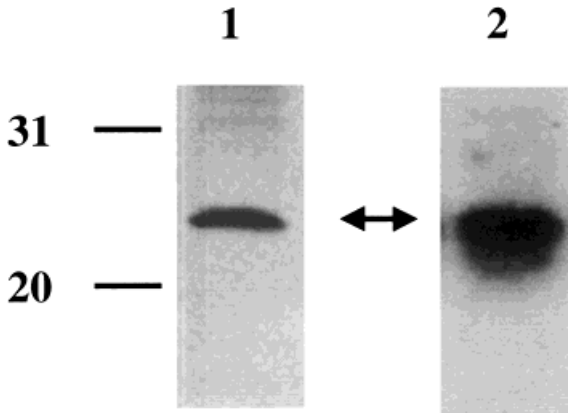

Fig. 4. Western blot with Ran antibody shown in **lane 1**. Overlay blot with [α^{32}P]-GTP performed on the same piece of nitrocellulose shown in **lane 2**. The experiment was repeated a total of three times using three separate placentae with similar results.

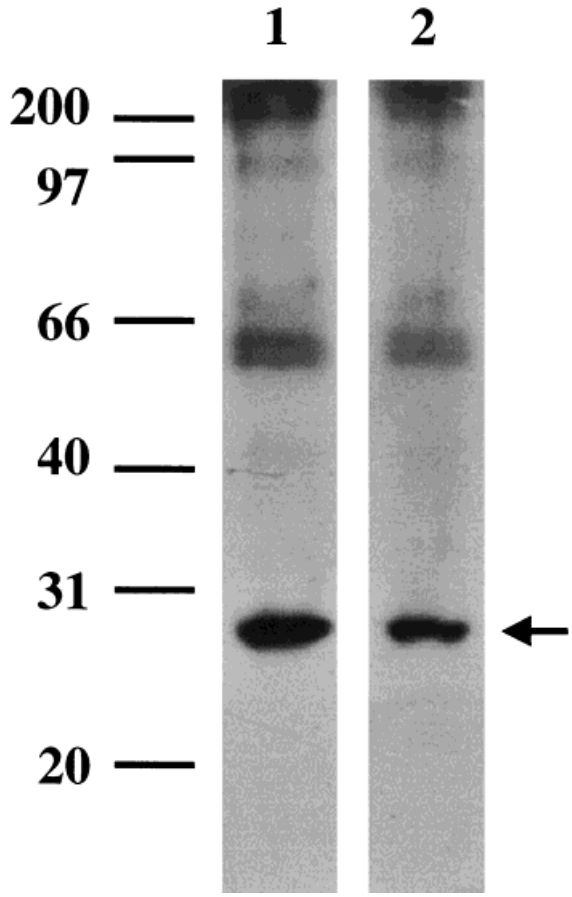

Fig. 5. Ran immunoreactivity in proteins from (**1**) mitochondria and (**2**) nuclei. The experiment was repeated a total of three times using three separate placentae with similar results.

The GTP-binding protein Ran was first identified in isolated rat liver nuclear envelopes by photo-affinity labelling with [α^{32}P]-GTP [Seydel and Gerace, 1991]. In a range of tissues not including placenta, Ran has been found to be an essential factor in import of proteins containing a nuclear localization sequence into the nucleus [Melchioir et al., 1993; Moore and Blobel, 1994]. The current nuclear import model [Cole and Hammell, 1998] suggests that import complexes are formed when Ran is primarily bound to GDP. This complex is then transported through the nuclear pore complex (NPC). Because Ran has been shown to be so important for nuclei function in other tissues and species we determined whether Ran was present in the nuclei of human placentae as discussed below.

Using antisera to Ran, a major band was identified at 27 kDa (Fig. 4). It appears possible that the 27 kDa [α^{32}P]-GTP binding protein is Ran and future studies will be needed to investigate this possibility. It will also be interesting to determine in future studies whether Ran is functioning to import biomolecules through the nuclear membrane in human placenta. There have been very few studies that have determined whether there are GTP-binding proteins other than Ran in the nucleus, in one other study using overlay blot Bhullar et al. [1999] have identified 25 and 26 kDa proteins that bind [α^{32}P]-GTP. Bhullar et al. [1999] also used Ran antisera to detect a major protein of approximately 26 kDa as well as a minor band of 32 kDa. Photoaffinity labelling has been used to detect an 80 kDa GTP-binding protein in the nuclei of rabbit liver nuclei [Singh et al., 1995]. In the present study we show that in addition to the 27 kDa protein, nuclei from human placenta contain a 24 kDa GTP-binding protein that does not cross-react with a antibody to Ran (Fig. 4). It will be important in future studies to determine the biological properties of the 24 kDa protein, and whether it shows structural similarities to known small GTP-binding proteins.

Interestingly, Ran was also identified in mitochondria from the human placenta (Fig. 5). Mitochondria have been shown to contain a GTP-binding protein of approximately 27 kDa [Thomson et al., 1998], however, prior to the present report the identity of this protein was not known. The mitochondrion shares some common features with the nucleus, both have two membranes and import biological molecules. Ran has been shown to function in import functions in the nucleus and it has been proposed that in the mitochondria GTP-binding

proteins modulate the import of proteins and cholesterol through outer and inner membrane contact points. This poses the question, does mitochondrial Ran regulate the mitochondrial import of biological molecules?

The presence of GTP-binding proteins in the nucleus of human placental cells raises numerous intriguing possibilities. The 24 and 27 kDa proteins may function to regulate protein synthesis in the organ and may control the growth and development and function of the placenta. As such these proteins may be a vital part of the myriad of placental functions which supports the growth and development of the fetus.

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