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# Human mitochondrial import receptor, Tom20p. Use of glutathione to reveal specific interactions between Tom20-glutathione *S*-transferase and mitochondrial precursor proteins

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**Abstract The cytosolic domain of the human mitochondrial protein import receptor, hTom20, has been expressed as a fusion protein with glutathione *S*-transferase (GST) in bacteria and the purified protein immobilized on Sepharose beads. To discriminate between specific binding of precursor proteins with the receptor and non-specific binding, precursors were recovered as a complex with GST-hTom20 following competitive elution from the beads with reduced glutathione. Here, we describe the specificity of this assay and demonstrate that the cytosolic domain of hTom20 interacts directly with the transcription-translation product of precursor proteins that bear a diverse array of targeting signals. Such proteins include a matrix protein (pODHFR), a polytopic integral protein of the inner membrane (uncoupling protein), a β-barrel protein of the outer membrane (VDAC/porin) as well as bitopic integral proteins which are inserted into the outer membrane by either an $NH_2$-terminal or COOH-terminal signal anchor sequence (yTom70(1–29)DHFR and Bcl-2, respectively).**



*Key words:* Mitochondria; Protein import; Tom20; Precursor protein

## 1. Introduction

Import of proteins into the mitochondrion is of immense importance for the biogenesis of the organelle, since greater than 95% of its resident proteins are encoded by nuclear genes. These precursor proteins are then targeted to the organelle as the result of specific interactions with receptor proteins located in the outer membrane (see [1–4] for review). Specifically, the receptor component hTom20 contributes to the import of a broad range of precursor proteins [5–7]. How this interaction [8,9] is achieved is thus crucial for understanding the import pathway. To date, many techniques have been developed to study protein-protein interactions, including co-immunoprecipitation [10], chemical cross-linking [11], and the yeast two-hybrid system [12]. Additionally, interactions can be studied using purified components obtained following expression of recombinant proteins in bacteria. Often, the proteins are expressed as fusion proteins that are specifically designed to facilitate binding to an insoluble support. While such a strategy is extremely useful for subsequent analyses of protein-protein interactions, it cannot be used in situations where the ligand exhibits non-specific binding to the affinity matrix. In preliminary analyses, we found this to be particularly true for mitochondrial precursor proteins.

To overcome this problem, we describe a simple, rapid and sensitive method for the study of interactions between the human import receptor, hTom20, and mitochondrial precursor proteins. This technique requires reagents that are relatively easy to generate. Specifically, a fusion protein has been generated between GST and the cytosolic domain of hTom20, and non-covalently bound to glutathione Sepharose. The beads are then incubated with a variety of test precursor proteins that are synthesized in a coupled in vitro transcription-translation system in the presence of [$^{35}$S]methionine. The identity of specific protein-protein interactions are revealed on SDS-PAGE by the presence of the radiolabelled proteins that have been competitively eluted with reduced glutathione. Using this approach, we demonstrate that hTom20 selectively and specifically interacts with a diverse array of mitochondrial precursor proteins

## 2. Materials and methods

### 2.1. Materials

All restriction enzymes were from New England Biolab, Inc., pGEX-2T and glutathione Sepharose 4B from Pharmacia Biotech, Inc., Topp2 cells from Stratagene, and GSH from Boehringer Mannheim GmbH.

### 2.2. Expression and purification of bacterial expressed GST and GST-Δ30hTom20

*E. coli* Topp2 cells were transformed with pGEX-2T encoding either GST or GST fused in-frame to amino acids 30–145 of human Tom20 [13]. Cells were grown to 0.6 $A_{600\ nm}$ and 1.0 mM isopropylthiogalactoside was added to the incubation medium. After 90 min, cells from 50-ml aliquots of the culture were recovered by centrifugation at 2700×*g* for 15 min at 4°C, suspended in 3.0 ml extraction medium (32 mM KCl, 0.8 mM Mg acetate, 15% (v/v) glycerol, and 4.0 mM HEPES, pH 7.5), sonicated 5×15 s with a small probe sonicator (Sonic dismembrator-Artek Systems Corporation; Fisher Scientific) operating at setting 6.0 at 4°C, and centrifuged at 17000×*g* for 15 min. The supernatant was incubated for 90 min at room temperature with 2 ml 50% glutathione Sepharose 4B, washed 2 times with extraction medium, and finally suspended and stored at 4°C in 3 ml extraction medium. To determine the amount of GST and GST-Δ30hTom20 on the beads, a small aliquot was incubated with an equal volume of 10 mM GSH in 50 mM Tris-HCl, pH 8.0, the protein recovered following centrifugation, and quantified using the BioRad protein assay. Glutathione Sepharose 4B was added to equalize the amount of protein/amount of Sepharose matrix used in the binding experiment.

### 2.3. In vitro translation of the preproteins

The SP64 plasmid containing the cDNA of pODHFR [14], yTom70(1–29)DHFR [15], UCP [16], and VDAC [17] were linearized by digesting with *Eco*RI or with *Pvu*II (VDAC). 6 μg of linearized

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*Abbreviations:* GSH, reduced glutathione; GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UCP, uncoupling protein; VDAC, voltage dependent anion channel


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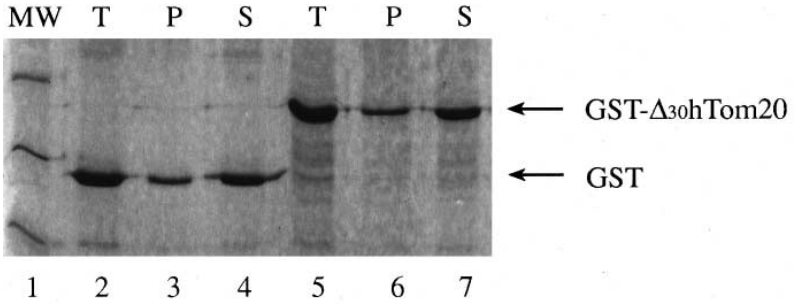


Fig. 1. Specific and reproducible elution of GST and GST-$\Delta_{30}$hTom20 with GSH. A Coomassie blue stained 12% SDS polyacrylamide gel revealing total amount of GST (lane 2) or GST-$\Delta_{30}$hTom20 (lane 5) that had been eluted by GSH (lanes 4 and 7). The remaining GST (lane 3) and GST-$\Delta_{30}$hTom20 (lane 6) still bound to the Sepharose 4B matrix is shown.

vector were diluted in 40 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 4 mM spermidine, 10 mM DTT, 10 mM NaCl, 50 μg/μl BSA, 0.5 mM of each NTP, 0.05 mM $m^7$GpppG-cap, 121 units Sp6-RNA polymerase and 117 units RNase inhibitor and incubated for 1 h at 37°C. The plasmid pBluescript $KS^-$ containing the cDNA of Bcl-2 [18] was linearized by digesting with *Pst*I. 6 μg of the linearized vector were diluted in 40 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 2 mM spermidine, 25 mM DTT, 10 mM NaCl, 0.4 mM of each NTP, 0.05 mM $m^7$GpppG-cap, 171 units T3-RNA polymerase and 58 units RNase inhibitor and also incubated for 1 h at 37°C. The RNA was precipitated and resuspended in 150 μl DEPC-$H_2O$. 2.5 μl RNA was added to 17.5 μl rabbit reticulocyte lysate, 1 μl amino acid mix without methionine, 1 μl [$^{35}$S]methionine and 3 μl DEPC-$H_2O$ and incubated for 30 min at 30°C.

*2.4. Binding of mitochondrial precursor proteins*

Binding reactions contained 10 μl suspended GST- or GST-Δ30hTom20-saturated Sepharose 4B, 38 μl extraction medium with or without additives (see figure legends), and 2 μl reticulocyte lysate translation product labeled with [$^{35}$S]methionine. The final concentration of GST and GST-Δ30hTom20 was 500 nM. After shaking for 20 min at room temperature, the reaction was stopped upon addition of ice-cold extraction medium and the beads immediately collected following centrifugation at 12000×*g* for 5 min at 4°C. The pellet was washed 2 times with 1.0 ml of the same medium, all excess fluid removed using a 10 μl Hamilton syringe, and the dried pellet resuspended in 20 μl 10 mM GSH and 50 mM Tris-HCl, pH 8.0, and incubated at room temperature for 30 min. The supernatant was recovered and 15 μl processed for SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250, fluorographed, and autoradiographic bands quantified with a Fuji Phosphor-imaging system (Fujix BAS 2000).

## 3. Results and discussion

*3.1. Specificity of interaction of the GST-fused cytosolic domain of hTom20 with precursor protein*

The cytosolic domain of hTom20 (amino acids 30–145) [13] was fused in-frame to GST and the purified hybrid protein (designated GST-$\Delta_{30}$hTom20) coupled to Sepharose 4B as a preliminary to examining interactions with precursor proteins. Conditions for the latter were established for pODHFR, a model protein comprised of the matrix targeting signal of pre-ornithine carbamyl transferase (amino acids 1–38) fused in-frame to amino acids 4–188 of dihydrofolate reductase [14]. Because the use of reduced glutathione (GSH) was predicted to be important to discriminate between specific vs. non-specific binding, it was first important to determine that GST and GST-$\Delta_{30}$hTom20 were competitively released by this agent from the glutathione Sepharose column to a similar extent.

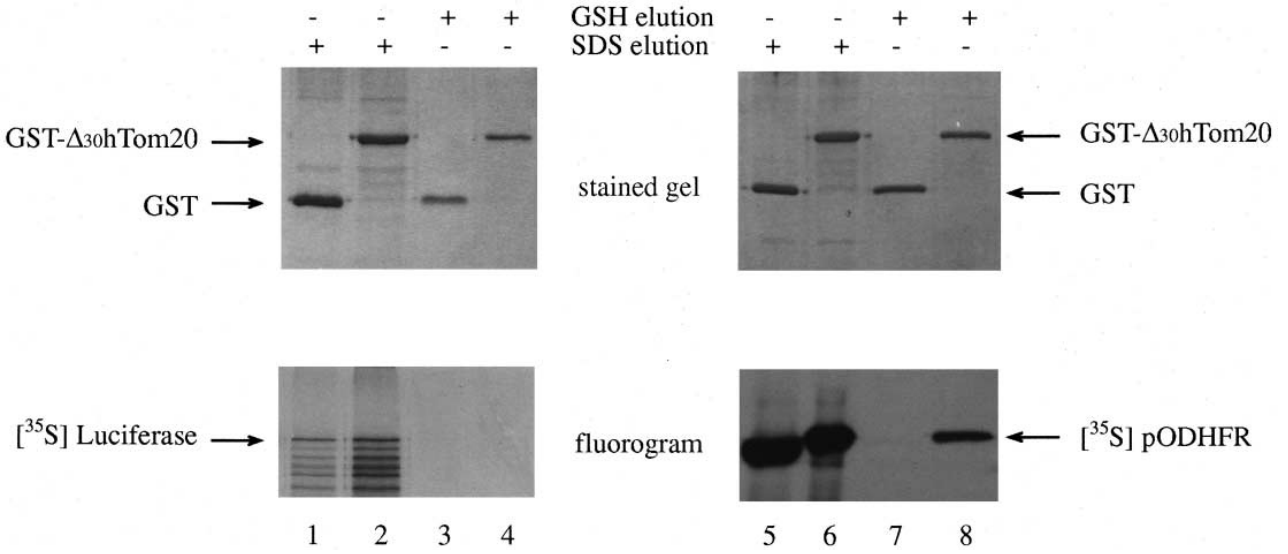


Fig. 2. Elution with GSH allows the determination of specific binding of precursor proteins to hTom20. GST- (lanes 1, 3, 5 and 7) or GST-$\Delta_{30}$hTom20- (lanes 2, 4, 6 and 8) bound Sepharose beads were incubated with [$^{35}$S]luciferase (lanes 1–4) or [$^{35}$S]pODHFR (lanes 5–8) as described in Section 2. Protein was released from the beads by incubation in either 2% SDS (lanes 1 and 2, and 5 and 6) or in 10 mM GSH (lanes 3 and 4, and 7 and 8). The corresponding 12% SDS acrylamide gel is shown as both a Coomassie blue stained gel (upper panel) or fluorogram (lower panel).

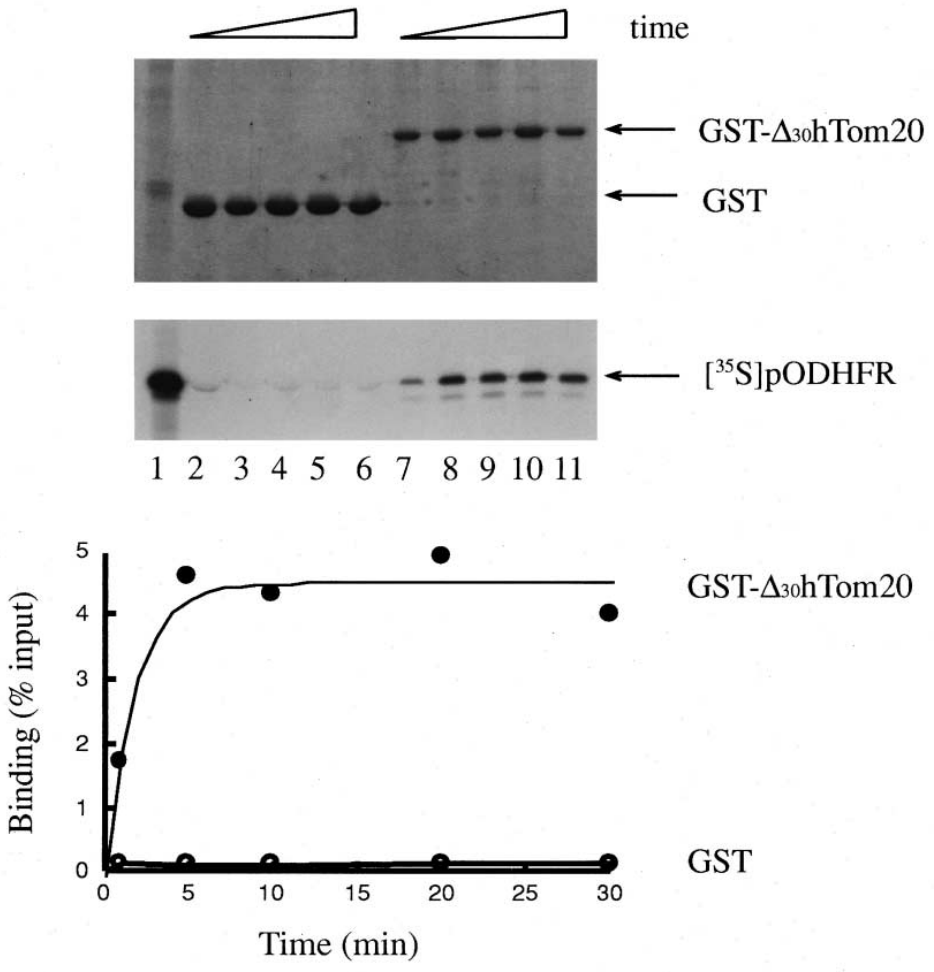


Fig. 3. Time course of interaction between GST-$\Delta_{30}$hTom20 and pODHFR. Binding reactions between [$^{35}$S]pODHFR (lane 1 represents 10% of input precursor protein) and either GST (lanes 2–6), or GST-$\Delta_{30}$hTom20 (lanes 7–11) were done essentially as described in Section 2 except that the reactions were performed on ice for 1 min, 5 min, 10 min, 20 min and 30 min. The upper panel represents the Coomassie blue stained gel as a control for fusion protein input. The middle panel represents the fluorogram of the same gel. The lower panel represents the quantitation of this particular experiment displayed as a line graph.

That this was the case is shown in Fig. 1. Sepharose beads containing equal amounts of GST and GST-$\Delta_{30}$hTom20 were treated with 10 mM GSH, resulting in the release of about 80% of the protein in both cases.

Fig. 2 compares the binding of pODHFR and a control cytosolic protein, luciferase, to GST- and GST-$\Delta_{30}$hTom20-Sepharose, followed by elution in medium containing either 2% SDS or 10 mM GSH. As judged by SDS elution of the Sepharose beads, the two proteins showed no preference for either GST or GST-$\Delta_{30}$hTom20, reflecting a high degree of non-specific binding (Fig. 2, lower panel, lanes 1 and 2, and 5 and 6). However, the underlying specific interactions of pODHFR with GST-$\Delta_{30}$hTom20 were clearly revealed by eluting with 10 mM GSH (lower panel, compare lanes 7 and 8). In contrast, no pODHFR was released from GST-Sepharose (lower panel, lane 7), indicating that the non-specific component of bound precursor was due exclusively to interactions with the beads and not with GST. Importantly, GSH did not release luciferase from either GST- or GST-$\Delta_{30}$hTom20-Sepharose (lower panel, lanes 3 and 4). Also, binding of pODHFR to GST-$\Delta_{30}$hTom20 was dependent on the presence of the matrix targeting sequence since pODHFR, but not DHFR, was found to interact with the receptor (unpublished results).

A time course for binding of pODHFR to GST-$\Delta_{30}$hTom20-Sepharose, as revealed by GSH elution, is shown in Fig. 3. Under the conditions employed, binding was rapid and complete within 5 min at 0°C. At room temperature, maximum binding occurred almost immediately. Importantly, however, GSH-sensitive binding to GST-Sepharose was not observed, even for binding periods up to 30 min (Fig. 3). The rapid binding of pODHFR to GST-$\Delta_{30}$hTom20-Sepharose is consistent with the finding that interaction of pODHFR with Tom20 in intact mitochondria is not a rate limiting step during import [17].

Finally, however, care must be exercised with respect to the composition of the binding reaction, since the interaction of GST to glutathione Sepharose is labile under certain conditions. Nevertheless, should this situation arise, it is readily revealed by analyzing the amount of GST-$\Delta_{30}$hTom20 that is liberated. In the example that is shown in Fig. 4 (right panel, stained gel), $FeCl_3$ released all of the fusion protein during the wash stage and, therefore, this reagent cannot be used to investigate its effects on receptor-protein interactions.

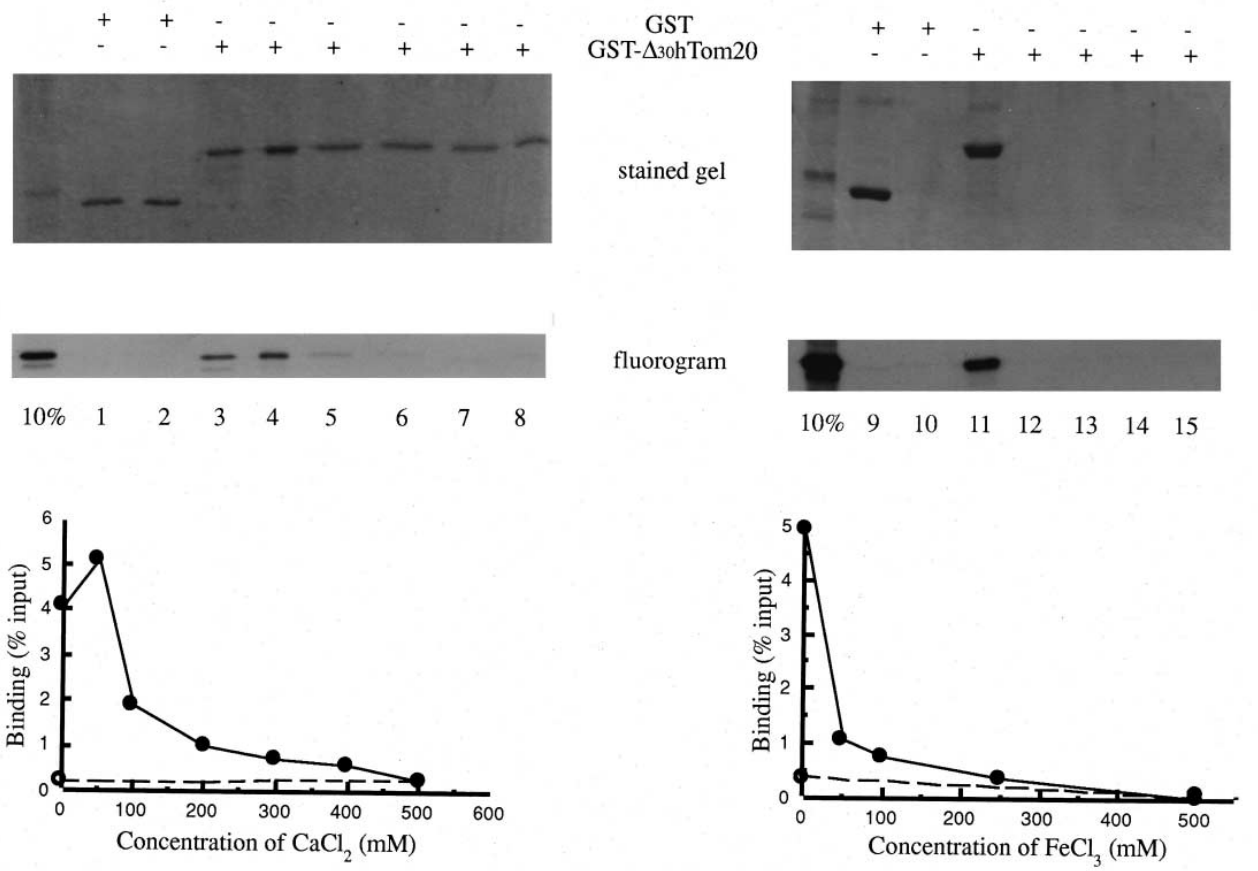


Fig. 4. Components in reaction affect some GST-glutathione Sepharose 4B interactions. Binding of [$^{35}$S]pODHFR (10% represents 10% of input precursor protein) to either GST (lanes 1 and 2, and 9 and 10), or GST-$\Delta_{30}$hTom20 (lanes 3 and 8, and 11 and 15) were done essentially as described in Section 2 except increasing concentrations of $CaCl_2$ (left panel) or $FeCl_3$ (right panel) were included. The upper panel represents the Coomassie blue stained gel. The middle panel represents the fluorogram of the same gel. The lower panel represents the quantitation of this particular experiment, displayed as a line graph.

$CaCl_2$, on the other hand, is permissible, and the results show that binding of pODHFR to GST-$\Delta_{30}$hTom20 is inhibited at high concentrations of this salt (Fig. 4, left panel).

*3.2. A wide spectrum of precursor proteins interact with Tom20*

Previous studies have shown that antibodies against hTom20 can block import of a diverse set of precursor proteins when mitochondria are pre-incubated with the antibody in vitro [13,17]. These include proteins targeted to the matrix via a matrix targeting signal (pODHFR) [14], to the inner membrane via multi-spanning domains (uncoupling protein (UCP), a homolog of the ADP/ATP carrier [19]), and to the outer membrane by both $NH_2$-terminal and COOH-terminal signal anchor sequences (yTom70 [15] and Bcl-2 [18], respectively) or by signals within the β-barrel protein, VDAC, the mammalian homolog of fungal porin [20,21]. Whether or not all of these proteins interact with Tom20 during import, or whether the observed inhibition of their import by antibody results from steric interference of the receptor complex as a whole, has not previously been addressed. To resolve this question, the various precursors were examined for their ability to interact directly with GST-$\Delta_{30}$hTom20 (Fig. 5). All precursors examined showed specific interaction, albeit to different extents. For example, UCP was the lowest, with ~1% of input demonstrating specific binding to the receptor.

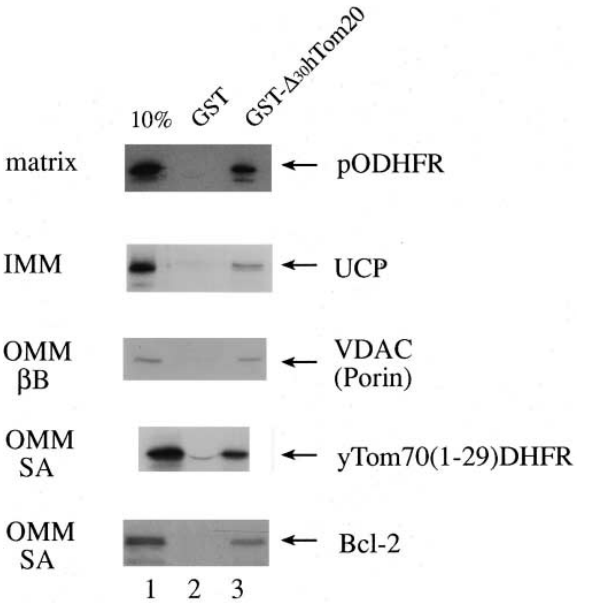


Fig. 5. Identification of the interaction of hTom20 to different preproteins. The interaction was studied as described in Section 2. GST (lane 2) or GST-$\Delta_{30}$hTom20 (lane 3) were incubated with in vitro translated pODHFR, UCP, VDAC, yTom70(1-29)DHFR, and Bcl-2. Lane 1 represents 10% of the input of each preprotein.

### 3.3. Conclusions

We have developed a system to examine interactions of precursor proteins with the cytosolic domain of the mitochondrial import receptor, hTom20. The use of GSH to liberate precursor-receptor complexes was key to discriminating between specific interactions with the receptor and non-specific binding of the precursors to the immobilization matrix, Sepharose 4B. Several criteria must be fulfilled, however, to validate the assay. For example, GSH must liberate equivalent amounts of GST and GST-receptor. This is easy to monitor by subsequent SDS PAGE and protein staining. Also, the system is incompatible with conditions that might lead to GSH-independent release of GST-receptor (e.g. buffers, salts, etc.). Once validated the assay provides a simple method to measure receptor-precursor interactions. Here, we have demonstrated that hTom20 interacts with a diverse class of precursor proteins.

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