Endocrine (1995) 3. 399-408
© 1995 Stockton Press All rights reserved 0969-711X/95 $12.00

Islet expression of Rhombotin and Isl-1 suggests cell type specific exposure of LIM-domain epitopes

Kaare Lund[1,3], Jacob S. Petersen[1], Jan Jensen[1], Niels Blume[1], Thomas Edlund[2], Stefan Thor[2] & Ole D. Madsen[1]

[1]*Hagedorn Research Institute, Niels Steensensvej 6, DK2820 Gentofte, Denmark;* [2]*Department of Microbiology, University of Umeå, S-901 87 Umeå, Sweden*

The homeodomain protein Isl-1 and the proto-oncogene Rhombotin (a LIM-only protein), share a double zinc-binding LIM domain and have both been implicated in neural and possibly endocrine development. Isl-1 is expressed in all endocrine cell-types of the islet of Langerhans while Rhombotin mRNA expression was reported in rat insulinoma cells. We have cloned and sequenced Rhombotin cDNA from rat insulinoma (99.4% identical to human and mouse sequences) and demonstrate that it is expressed in normal islets, intestinal tissue, and testis, in addition to the brain; but absent in all other organs tested. Rhombotin mRNA is expressed in phenotypically distinct islet tumours (α-, β, and δ-tumours) at levels comparable to that of normal islets. Antisera raised against two distinct epitopes contained within a short synthetic peptide representing part of the N-terminal LIM domain of Rhombotin surprisingly stain α- and δ-cells, respectively, on sections of rat pancreas. Rhombotin is undetectable by immunocytochemistry using LIM-domain antisera on intact monolayer islet tumor cells or transfected fibroblasts while readily detectable when equipped with a FLAG epitope, as detected with FLAG antiserum. In contrast, recombinant FLAG-Rhombotin is efficiently recognised by Western blotting or immunoprecipitation with all LIM-specific antisera. Almost identical results were obtained with LIM-specific versus homeodomain/C-terminal Isl-1 antisera staining α-cell cytoplasm or all islet nuclei, respectively. We conclude that Rhombotin in addition to Isl-1 is expressed in the islet of Langerhans and propose that the differential staining patterns obtained with antisera towards the LIM domains versus flanking epitopes of both proteins reflect (1) cell-specific protein-protein interactions of these domains or, alternatively, (2) islet cell type specific expression of novel homologous LIM domain proteins.

Keywords: Islets of Langerhans; gene expression; LIM domain proteins; RBTN1; protein dimers; epitope masking

Introduction

Rhombotin and Isl-1 belong to the family of LIM proteins which is characterized by the presence of one or more highly conserved zinc-binding cysteine/histidine motifs (Way & Chalfie, 1988; Freyd *et al.*, 1990; Karlsson, *et al.*, 1990; Sanchez-Garcia & Rabbitts, 1994). Isl-1 belongs to the subfamily of LIM-homeodomain transcription factors where the DNA-binding homeodomain is flanked N-terminally by two LIM domains. Although cloned from a pancreatic β-cell cDNA library Isl-1 has been implicated in motor neuron specification during early brain development (Thor *et al.*, 1991; Ericson *et al.*, 1992). Other LIM-homeodomain proteins such as the Lin-11 and Mec-3 from *C. elegans* are involved in determination of cell lineage, specifying vulval precursor cells and mechanosensory neurons, respectively (Way & Chalfie *et al.*, 1988; Freyd *et al.*, 1990), and the *Drosophila* protein Apterous, is required for imaginal disc development and the development of a subset of embryonic muscles (Bourgouin *et al.*, 1992; Cohen *et al.*, 1992).

Rhombotin is the product of a translocated T-cell proto oncogene which is a member of a small family comprising three related genes designated *rhombotin* (recently renamed *RBTN1*), *RBTN2* and *RBTN3* (Boehm *et al.*, 1991; Royer-Pokora *et al.*, 1991; Foroni *et al.*, 1992). All three genes contain two repeated LIM domains, as does e.g. Isl-1, but have no homeodomain or any conserved transcription promoting regions, and are as such known as LIM-only proteins (McGuire *et al.*, 1989; Boehm *et al.*, 1990b). Homologues of the *rhombotin* gene have been isolated from evolutionarily distinct species like man and mouse (Boehm *et al.*, 1990b; McGuire *et al.*, 1991) and Drosophila (Boehm *et al.*, 1990a), and the sequence has proven to be highly conserved, indicating an important function of this gene as well as of the LIM motif itself (Sánchez-García *et al.*, 1993). The *rhombotin* and *RBTN3* genes are located on different chromosomes but show overlapping expression patterns in the developing mouse brain (Foroni *et al.*, 1992). Rhombotin expression is thus reported to be restricted mainly to the central nervous system where it is differentially expressed from alternative promoters in mice during embryonic development (Greenberg *et al.*, 1990; Foroni *et al.*, 1992), but sites of high level expression also include cell lines of neuroendocrine origin, including transformed rat islet cells (Boehm *et al.*, 1990b). RBTN2 is expressed more ubiquitously (Boehm *et al.*, 1991; Foroni *et al.*, 1992) and has recently been shown to occupy a key role in erythropoeisis, as total ablation of the erythroid lineage was observed in RBTN2 null-mutant mice (Warren *et al.*, 1994). The *rhombotin* and *RBTN2* genes were revealed as protooncogenes in man by the consistent correlation of ectopic expression after translocation to the T-cell receptor locus of either of the two genes in immature lymphoid cells leading to acute T-cell lymphoid leukaemia (T-ALL) (McGuire *et al.*, 1989; Boehm *et al.*, 1990b). A similar effect has been demonstrated in transgenic mice where animals that expressed either *rhombotin* or *RBTN2*, under control of a lymphoid cell-specific promoter, developed leukaemia with frequencies depending on the level of expression of the transgene (Fisch *et al.*, 1992; McGuire *et al.*, 1992). In contrast, targeted expression of Rhombotin to the pancreatic β-cell under the control of the insulin promoter did not give rise to tumor formation in transgenic mice (Fisch *et al.*, 1992). These data emphasises that LIM-only proteins can be potent regulators of gene expression despite the lack of DNA binding and transcription activating domains. Moreover, ectopic expression of rhombotin was recently shown to promote myogenic differentiation, a process normally controlled by the muscle specific LIM protein, MLP, which is another LIM-only protein (Arber *et al.*, 1994).

Recent studies suggest a functional role of the LIM domains in protein-dimer formation (Rabbitts & Boehm,

Correspondence: O.D. Madsen
[3]Current address: Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637.
Received 7 October 1994; accepted 22 February 1995

1990; Sadler *et al.*, 1992; Schmeichel & Beckerle, 1994) and direct association between RBTN2 with the basic helix-loop-helix protein TAL1 in erythroid cells has recently been demonstrated (Valge-Archer *et al.*, 1994). Among the LIM-homeobox proteins protein-dimer formation may also be important for correct function as many have been shown to act in synergy with other transcription factors. Isl-1 interacts with the cAMP responsive factor (CREB) on the somatostatin gene enhancer (Leonard *et al.*, 1992), MEC-3 regulates the activity of its own gene in synergy with the POU-domain protein UNC-86 for maximal effect (Xue *et al.*, 1992) and the LIM domain of Lmx-1 is necessary for the synergistic effect with Pan-1 on insulin gene activation (German *et al.*, 1992). Isl-1-like immunoreactivity has previously been demonstrated in all four endocrine cell types of the islet of Langerhans (Dong *et al.*, 1991; Thor *et al.*, 1991), while Rhombotin mRNA has been detected in rat insulinoma cells (Boehm *et al.*, 1990b). In this study we have cloned rat islet Rhombotin cDNA and demonstrate Rhombotin mRNA expression in normal islet cells as well as of phenotypically distinct islet tumors. In addition, we have raised and characterized antibodies against peptides specific for Rhombotin and Isl-1 LIM-domain sequences. The reaction pattern of these antisera could suggest that the LIM proteins in question are engaged in cell-type specific protein-protein interactions or alternatively, that hitherto undefined homologous LIM proteins are expressed in a cell type specific manner within the islet of Langerhans.

Results

Cloning of rat rhombotin from RIN-5AH cDNA

In order to study *rhombotin* expression in rat islets *rhombotin* cDNA was cloned by PCR from the rat insulinoma cell line RIN-5AH. The PCR primers used to amplify rat *rhombotin* were synthesised based on the human sequence. Primers were chosen to include the first ATG codon of exon 1a for the 5′-primer and the termination codon TAA for the 3′-primer (Figure 1). PCR products were either cloned into the HphI site of the pCR-I vector or were sequenced directly in order to obtain sequence information not biased by PCR-generated mutations which might prevail in individual clones. Throughout the coding region the sequence of rat *rhombotin* cDNA is very similar to both the mouse and human sequences (Figure 1A). Rat specific variations in the cDNA sequence occur in only five out of 471 positions and give in no instances rise to unique amino acid substitutions. Of the two differences in the amino acid sequence between the mouse and human proteins one from each is found in the rat. The sequences covered by the two PCR primers were verified by amplification and sequencing of the 5′-untranslated region of exon 1a with a second 5′-primer matching the human upstream sequence (Boehm *et al.*, 1990b) and by 3′-RACE with an internal gene specific primer and an oligo-$(dT)_{17}$-containing adapter primer (see Materials and methods).

Rhombotin mRNA expression profile

By semi-quantitative RT-PCR we analysed Rhombotin mRNA expression in several tissues including isolated rat islets as well as in the three distinct rat islet tumor phenotypes (glucagonoma, insulinoma and somatostatinoma) which are all derived from the pluripotent islet tumor line, MSL-G2 (Figure 2). We confirm the previously reported expression in brain and RIN cells, but significant levels were present in normal islets as well as in all islet derived tumor cells. Moreover, Rhombotin mRNA is expressed also in stomach and intestinal tissues as well as in testis while absent in lung, liver, kidney and spleen.

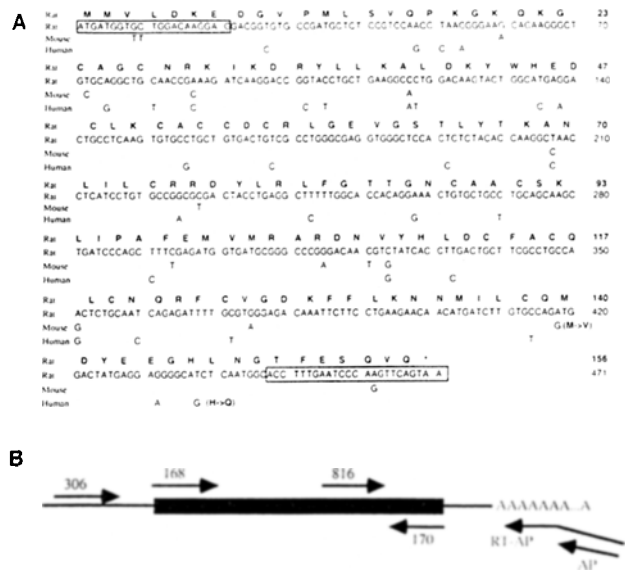


Figure 1 (**A**) Comparison of the rat *rhombotin* cDNA sequence, including the sequence of exon 1a, with the mouse and human sequences. Discrepancies between the mouse, human and rat sequence are listed below the rat sequence. The Rhombotin amino-acid sequence is conserved between the three species, except in position 140 and 146. Both these positions differ between mouse and human, but in the rat sequence residue in position 140 is a methionine, like in the human sequence, and the residue in position 146 is a histidine, like in the mouse sequence. The sequence corresponding to the annealing sites of the primers used to clone rat *rhombotin* cDNA are boxed. (**B**) Schematic representation of the position of the various PCR primers relative to the *rhombotin* cDNA that were designed in order to obtain sequence information of the entire coding region of rat rhombotin. RT-AP and AP denote primers used in the 3′-RACE reactions (see Materials and methods)

Characterisation of LIM-domain antisera towards recombinant Rhombotin and Isl-1 proteins

A series of antibodies were raised against synthetic peptides representing the C-terminal part of the LIM-domains in both Rhombotin and Isl-1. The specific LIM-domain epitopes chosen for antibody production were based on previous results obtained with the LIM-domain of CRIP (Madsen *et al* unpublished). The position of the different epitopes are outlined in Figure 3A and the sequence of the immunising peptides and corresponding antisera are listed in Figure 3B. The specificities of the various antisera were characterised by Western blotting using recombinant Isl-1 and a recombinant fusion protein consisting of Rhombotin with a short eight amino-acid epitope, FLAG, tagged to the N-terminus (Figure 4), both expressed in *E. coli*. Previously characterised antisera raised against the C-terminal region of recombinant Isl-1 (Thor *et al.*, 1991) and a monoclonal antibody, M2, against the FLAG sequence (Prickett *et al.*, 1989) were used as controls (Figure 3A and 4). All Rhombotin antisera (Ab1974, Ab1976 and Ab32) as well as the anti-FLAG M2 antibody yielded a single specific band of approximately 21 kDa on Western blots of FLAG/Rhombotin bacterial extracts, in accordance with the predicted size of the recombinant FLAG-Rhombotin fusion-protein (Figure 5 lanes 1–3, staining with antiserum no. 32 not shown). Only preabsorption with the immunising peptides removed reactivity towards the specific band indicating that the two antisera raised against the proximal (N-terminal) LIM-domain (Ab1974 and AB1976, lanes 4 and 5) as well as Ab32 against the distal (C-terminal) LIM-domain (not shown) are highly specific towards these epitopes. The M2 antibody staining was not affected by preabsorption with the two Rhombotin-LIM peptides (not shown). A similar pattern was seen for recombinant Isl-1 that

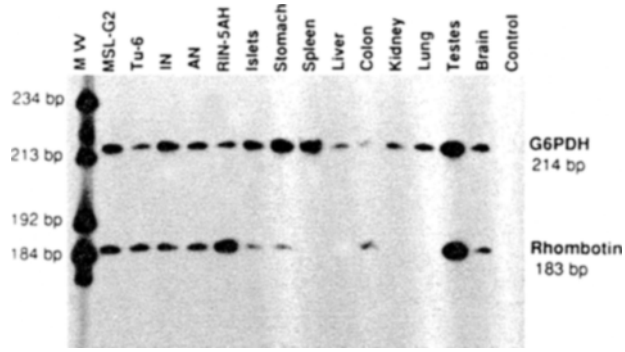


Figure 2 Semiquantitative RT-PCR based detection of Rhombotin mRNA using G6PDH as mRNA quality control (see Materials and methods). Amplification (25 cycles) was within the log-linear phase which allows semi-quantitative comparisons of Rhombotin/G6PDH expression levels among the various samples. MSL-G2 represents a pluripotent transformed rat islet culture from which a somatostatinoma (Tu6), an insulinoma (IN), and a glucagonoma (AN) was derived. As in RIN-cells (rat insulinoma cell line) and the previously mentioned islet tumors Rhombotin expression was detected in isolated islets, stomach, colon, testes and brain

Figure 3 (**A**) Schematic representation of the Isl-1 and Rhombotin proteins. Black boxes indicate the positions of the immunising peptides and protein fragments that were used to generate the antisera indicated. HOMEO denotes the Isl-1 homeo-domain. LIM 1 and LIM 2 denote the proximal and distal LIM domains, respectively. (**B**) Comparison of the peptides used to generate anti-Rhombotin and anti-Isl-1 LIM-domain-specific antisera. From the sequence comparison between Rhombotin (RBTN1) and RBTN3 it is evident that the resulting antisera would recognise both proteins. The CgB sequence representing a proteolytic fragment of chromogranin B [fragment 7 (Nielsen, *et al.*, 1991)] was used as an unrelated control peptide in preabsorption experiments. In the islet CgB is selectively expressed in α-cells and antisera against this sequence also produce a specific α-cell cytoplasmic staining

was recognised as a major band of predicted size on Western blots by both the LIM-specific and the C-terminal-specific anti-Isl-1 antisera (lanes 7 and 8). The reactivity of Ab1958 towards recombinant Isl-1 was abolished by preabsorption with the homologous immunising peptide (Figure 5 lanes 9 and 10) but not with homologous Rhombotin-peptides or an irrelevant chromogranin B-peptide.

Staining of rat islet sections with Rhombotin and Isl-1 specific antibodies

Unexpectedly, the two antisera, Ab1974 and Ab1976, raised against the same synthetic LIM-domain peptide of Rhombotin produced highly distinct stainings of rat islets. While Ab1974 stained all δ-cells on frozen sections then Ab1976 stained a subpopulation of α-cells on paraffin embedded sections (Figure 6). The cell specificity was confirmed by two-colour immunoflourescence staining where Ab1974 (in green) were combined with a monoclonal δ-cell specific rat autoantibody, CA812 (in red, Figure 6, panel E *vs.* F); and Ab1976 (in green) was combined with a monoclonal glucagon-specific antibody (in red, Figure 6, panel A *vs.* B). The highly distinct staining patterns obtained by the two antisera were completely abolished by preabsorption with the synthetic peptide (no. 189, Figure 2A) that was used for immunisation, whereas heterologous peptides including glucagon-or somatostatin-sequences had no effect (not shown). Ab32 raised against the homologous region of the second LIM domain of Rhombotin produced an α-cell staining pattern similar to that of Ab1976 (not shown). Like for the other LIM-specific antisera this staining pattern was highly specific, since it was only abolished by absorption with the immunising peptide.

Ab1958 raised against a peptide corresponding in sequence to a region in the distal Isl-1 LIM domain also produced an unexpected staining pattern on rat islet sections in the form of cytoplasmic staining of the entire peripheral islet α-cell population, as revealed by the double staining with a glucagon-specific antiserum (Figure 6G *vs.* H). In preabsorption experiments only the immunising peptide abolished staining, while the two Rhombotin LIM-domain peptides had no effect, eliminating the possibility of crossreactivity between the LIM-specific antisera. The staining pattern obtained with the LIM specific anti-Isl-1 antiserum is in contrast to that obtained by the anti-Isl-1 antibody raised against the entire homeodomain and C-terminal part of the protein, which has previously been reported to stain nuclei in

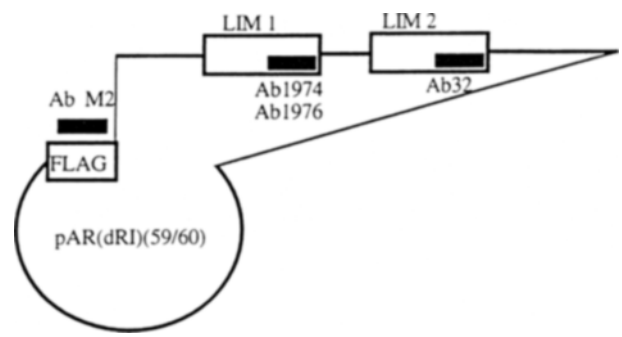


Figure 4 Map of the FLAG-Rhombotin *E. coli* expression vector. The FLAG sequence is a small octapeptide that was tagged to the recombinant protein as a distal epitope which can be recognised by a mouse monoclonal antibody, M2, independently of the exposure of the LIM specific epitope

all islet cell types (Thor *et al.*, 1991). This is shown in panel J as a dual exposure of a combined staining with the Isl-1 anti-C-terminal antibody (green) and the anti-glucagon antiserum (red). The staining patterns of the different anti-Rhombotin and anti-Isl-1 antisera are summarised in Table 1.

Detection of recombinant Isl-1 or Rhombotin expressed in transfected mammalian cells

Analysis of bacterially expressed recombinant proteins by Western blotting relies on interactions of the antibodies with denatured unmodified epitopes. In intact mammalian cells, however, proteins are subjects of posttranslational

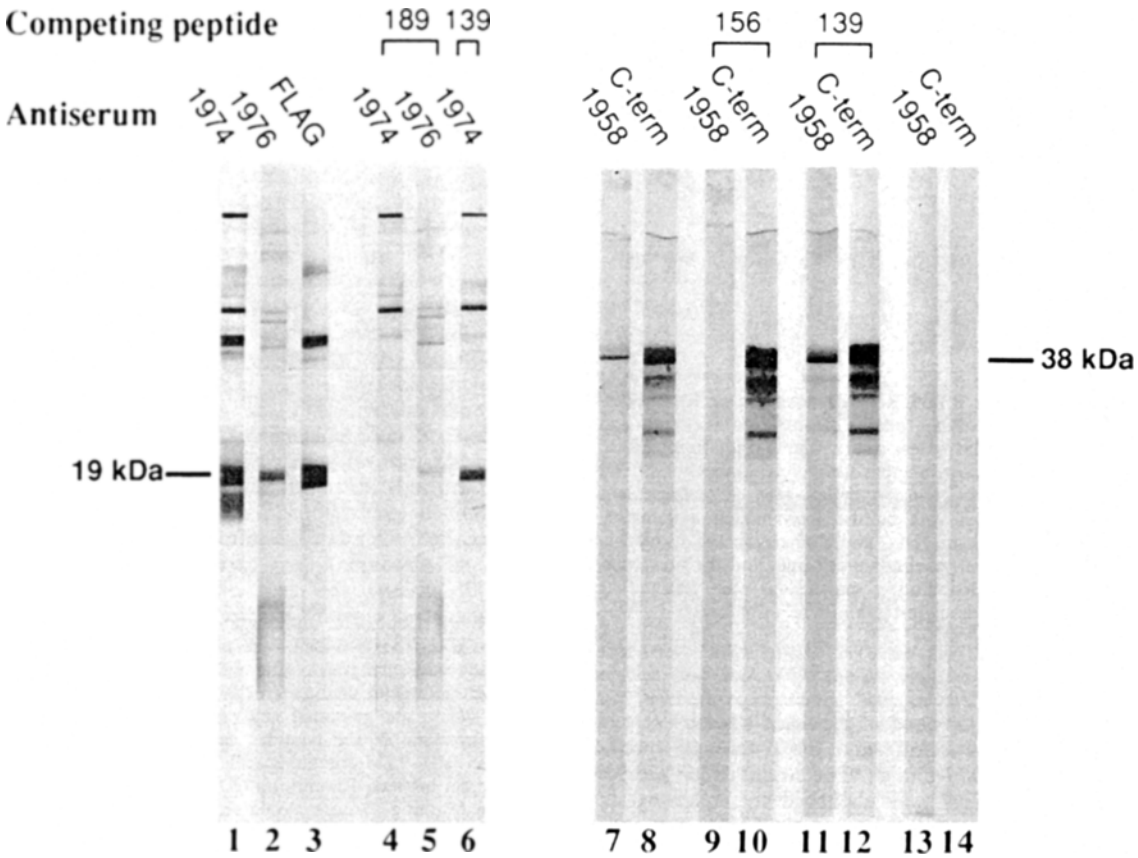


Figure 5 Western blot of bacterially expressed recombinant Isl-1 and FLAG-Rhombotin. FLAG-Rhombotin fusion protein was stained by the two anti-Rhombotin LIM-specific antisera Ab1974 and Ab1976 in addition to the monoclonal anti-FLAG antibody (lanes 1–6). Isl-1 was stained by the LIM-specific Ab1958 and the C-terminal specific anti-Isl-1 antibody (lanes 7–14). Both of the two LIM-specific anti-Rhombotin antibodies and the anti-FLAG antibody recognise one prominent band of expected size (approximately 19 kDa – indicated by the arrow, lane 1–3) which corresponds well with the size of the 18 kDa Rhombotin protein plus the weight of the small (0.96 kDa) FLAG epitope. The anti-Rhombotin antibodies specifically recognise the fusion protein, as only this band disappear (or is strongly reduced in intensity) after preincubation with the immunising peptide (lanes 4 and 5), while an irrelevant peptide has no effect (only shown for Ab1974, lane 6). The unspecific bands of higher molecular weights are thus not affected by preabsorption (lanes 1–6). The two anti-Isl-1 antibodies recognise the same 38 kDa species (lanes 7 and 8), and staining by the LIM-specific antibody can only be abolished by the immunising peptide (compare lanes 9 and 11). Additional bands (lane 8, 10, and 12) most likely represents proteolytic degradation of Isl-1. Some of these fragments harbour the LIM epitope (compare lane 7 and 8 or 11 and 12). Staining by the anti-Isl-1 C-terminal specific antibody was not affected by either peptide (lanes 10 and 12) as expected. Lanes 13 and 14 contain extract from mock-transfected bacteria. The antisera used in each case are indicated above the lanes, where Ab1974 and Ab1976 were raised against peptide 189 (Rhombotin LIM domain); FLAG is antibody M2 against the FLAG epitope; Ab1958 was raised against peptide 156 (Isl-1 LIM domain); and C-term. is the c-terminal specific Isl-1 antiserum. The immunising peptides, 189 and 156 were used in preabsorption experiments in combination with the irrelevant chromogranin B peptide, 139 (see Figure 3B for sequence information)

modifications, folding, dimer formation etc., and epitopes that might be accessible to the antibodies in Western blotting could be buried within the native protein in a manner concealing it from recognition by an antibody. The LIM-domain proteins are likely to rely on disulphide bridging and ligation of zinc and/or one or more iron-sulphur clusters (Li *et al.*, 1991; Michelsen *et al.*, 1993) in order to achieve correct folding. To test if the different antisera would recognise the recombinant proteins in their native state the FLAG-Rhombotin construct was cloned into the mammalian expression vector pcDNA1/neo, and transiently transfected into cos-7 cells. In a similar fashion Isl-1 was expressed transiently in cos-7 cells under control of an RSV promoter (Leonard *et al.*, 1992). Transfected cells were gently fixed in paraformaldehyde and stained with either Rhombotin anti-LIM antisera, Ab1974 and Ab1976, in addition to the anti-FLAG M2 monoclonal antibody (Figure 7A), or with the anti-Isl-1 LIM-specific peptide antibody Ab1958 in addition to the anti-Isl-1 C-terminal-specific antibody (Figure 7B and C). Expression of the FLAG-Rhombotin fusion protein in the cos-7 cells was evident from staining with the M2 antibody which labelled an expected fraction of the transfectants (Figure 7A) where nuclear as well as cytoplasmic staining was observed. No staining could be seen, however, with any of the anti-Rhombotin-LIM antisera (data not shown). This observation could be the result of protein degradation or premature termination of either transcription or translation of the transfected construct that would compromise the expression of the LIM-specific epitopes as the FLAG epitope is positioned N-terminally in the fusion protein. Therefore, protein was extracted from a pool of FLAG/Rhombotin-transfected cos-7 cells originating from the same transfection as the stained monolayer cells, and the fusion protein was immunoprecipitated with Ab1974 and Ab1976. A single prominent band of predicted size appeared on the autoradiogram with Ab1974 (Figure 7D) and less intense, but identical in size, with Ab1976 (not shown). The immunoprecipitated band co-migrated with that of immunoprecipitated *in vitro* translated FLAG/Rhombotin fusion protein (Figure 7D). The FLAG/Rhombotin fusion protein is thus expressed in

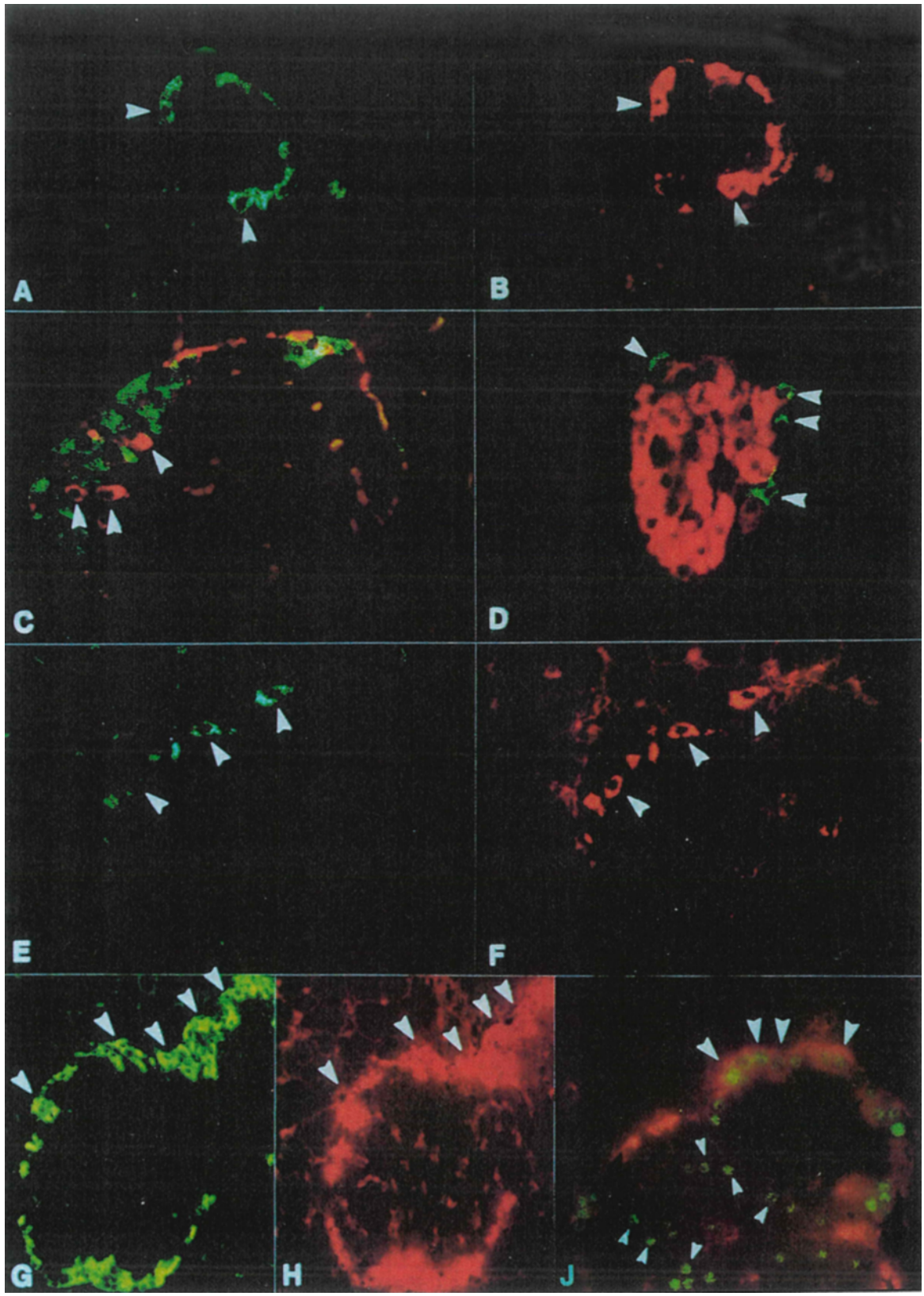


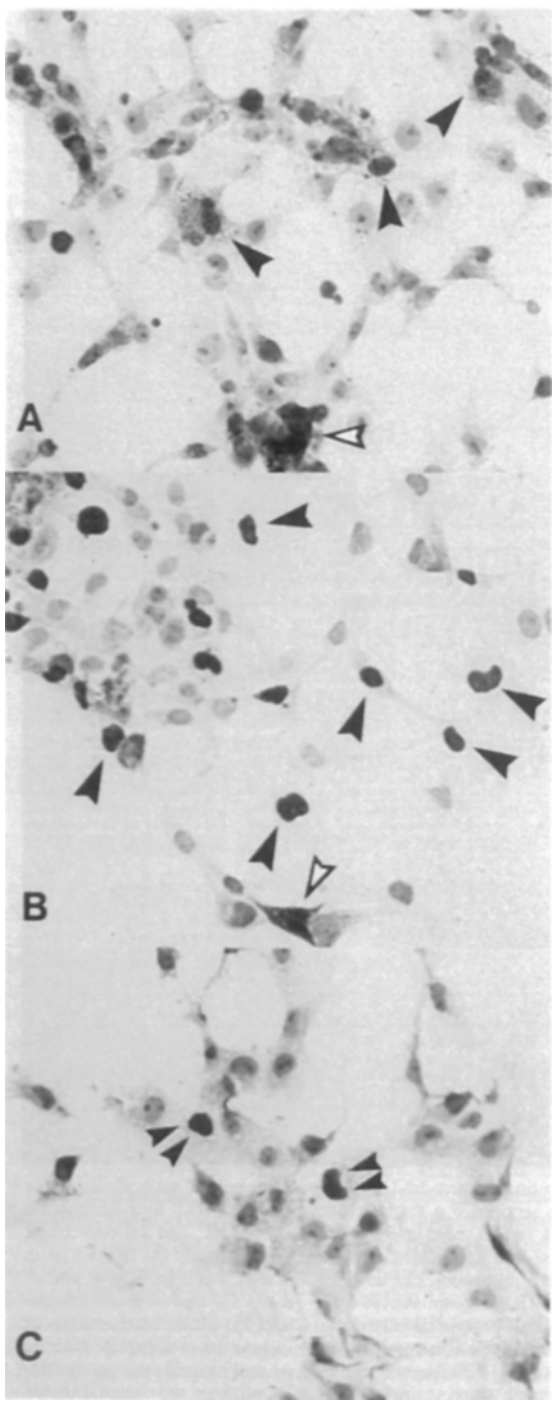
Figure 6 Immunocytochemistry on rat islet sections with anti-Rhombotin and anti-Isl-1 antisera compared to antibodies specific for different islet cell phenotypes. Panel A and panel B: same paraffin section of a rat islet double-stained with anti-Rhombotin Ab1976 (in green, **A**) and a monoclonal antibody towards glucagon (in red, **B**, note overlapping staining). (**C** and **D**) two-colour immunofluorescence labelling using Ab1976 (in green) in combination with a monoclonal somatostatin antibody (in red, **C**) or in combination with a monoclonal insulin antibody (in red, **D**). Ab1976 is thus specifically labelling the islet α-cell (**A**–**D**). **E** and **F**: same cryostat section of an unfixed rat islet double-stained with anti-Rhombotin Ab1974 (in green, **E**) and a δ-cell specific antiserum, CA812, (in red, **F**). The two antisera recognise the same subset of peripheral islet cells. (**G** and **H**) same frozen section of a rat islet double stained in green (**G**) with Ab1958 (anti Isl-1 LIM domain) and in red (**H**) for glucagon (note complete overlap indicating that Ab1958 reacts to Isl-1-like material in the α-cell cytoplasm). (**J**) double staining of a frozen unfixed islet section for glucagon (in red) and C-terminal specific Isl-1 immunoreactivity (in green) which stains the nuclei of all islet cell types. Limited glucagon diffusion is observed in frozen sections (red colour in **H** and **J**)

Table 1 Summary of immunocytochemical staining patterns produced by the anti-Rhombotin (Ab1974, Ab1976, and Ab32) and anti-Isl-1 specific antibodies (Ab1958 and anti-Isl-1 C-terminal) on sections of pancreatic rat islets

Antiserum	*Staining pattern*	*Subcellular localisation*
Ab1974	Peripheral islet cells Overlaps completely with δ-cells	Cytoplasm
Ab1976	Peripheral cells Overlaps completely with α-cells	Cytoplasm
Ab32	As Ab1976	Cytoplasm
Ab1958	As Ab1976 and Ab32	Cytoplasm
Anti-Isl-1 C-terminal	All islet cell types	Nuclei

intact cos-7 cells in a manner leaving the LIM-domain epitopes inaccessible for antibody recognition. A gentle lysis procedure unmasks the LIM-domain epitopes and the fusion protein is readily detected by immunoprecipitation. No additional bands were consistently co-precipitated with any of these antisera suggesting that the lysis procedure may dissociate potential heterodimers involving FLAG/Rhombotin. Similarly, preincubation of in vitro translated FLAG/Rhombotin with nuclear extract from Cos-7 cells prior to immunoprecipitation failed to reduce band intensity, which again may reflect sub-optimal conditions for detecting protein-dimer formation (Figure 7D)

A large fraction of the Isl-1-transfected Cos-7 cells were likewise stained with the anti-Isl-1 C-terminal-specific



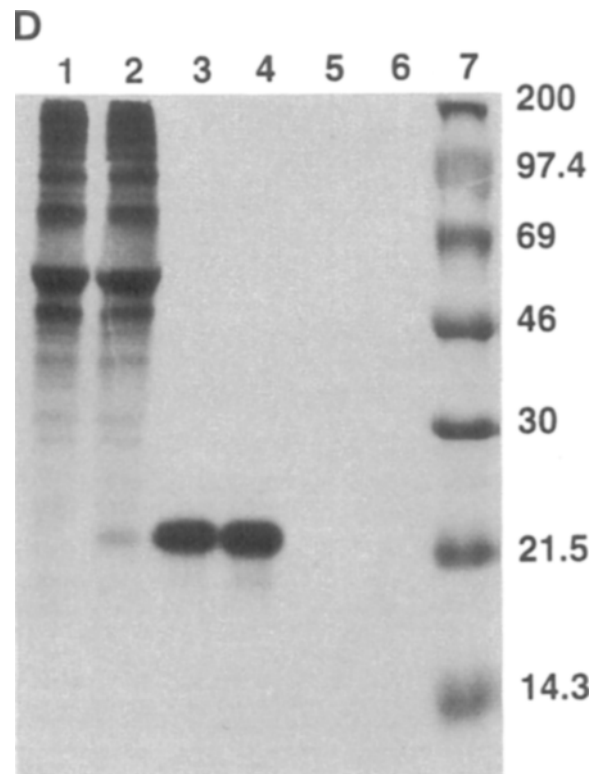


Figure 7 Transient expression of recombinant FLAG-Rhombotin or Isl-1 in Cos-7 cells. (**A**) Staining with the anti-FLAG antiserum showed both nuclear and cytoplasmic staining of the subpopulation of FLAG-Rhombotin-transfected cells (similar to the fraction of cells that were positive for luciferase produced by a co-transfected control plasmid – data not shown). Neither of the LIM-specific anti-Rhombotin antibodies were able to stain this sub-population of cells (not shown). (**B** and **C**) Isl-1-transfected cells were stained by the C-terminal Isl-1-specific antibody (**B**) in a frequency comparable to the luciferase control (not shown). The staining was localised to the nucleus in the vast majority of cells (filled arrow heads), but very few cells displayed also cytoplasmic staining (open arrow **B**). In comparison the LIM-specific Ab1958 only stained the nucleus of a very small fraction of transfected cells (selected area shown in **C**). (**D** Immunoprecipitation analysis for anti-rhombotin activity using Ab1974. Lane 1 and 2: mock-transfected versus FLAG-Rhombotin transfected cos-7 cells. Lane 3 and 4: *In vitro* translated FLAG-Rhombotin alone or preincubated with nuclear cos-7 cell extract, respectively. Lane 5 and 6: *In vitro* transcribed control template treated as lane 3 and 4. Lane 7 is molecular weight standards

antibody (Figure 7, B) indicating comparable transfection efficiency as with the FLAG/Rhombotin construct detected by the FLAG antiserum (compare Figure 7A, panel A and B). The staining was predominantly located in the nucleus while a few cells also showed cytoplasmic staining. In contrast, the Isl-1-LIM-specific Ab1958 (Figure 7C) stained only a very small subpopulation of these cells in parallel cultures from the same transfection (estimated to be less than 10%) again suggesting that the LIM-epitope is efficiently masked in the vast majority of the transfected cells.

Discussion

We have isolated and determined the sequence of Rhombotin from rat insulinoma cDNA and have demonstrated that it is also a product of the normal non-transformed islet of Langerhans as well as of phenotypically distinct α-, β- and δ-islet cell tumors all derived from common clonal origin (Madsen *et al.*, 1988; 1993a,b) where it is expressed at comparable levels. These data suggest that in contrast to the transforming potential when linked to the T-cell receptor locus in T-cell leukaemia in man (Boehm *et al.*, 1991; McGuire *et al.*, 1992), Rhombotin may not contribute to tumor formation in tissues where it is normally expressed. In accordance with these observations transgenic mice expressing Rhombotin under the control of the insulin promoter do not develop islet tumors (Fisch *et al.*, 1992). Rhombotin mRNA expression in stomach as well as intestinal tissue is interesting and could reflect a functional relationship with that of cysteine rich intestinal polypeptide (CRIP) (Birkenmeier & Gordon, 1986), another LIM-only protein involved in dietary zinc uptake (Hempe & Cousins, 1991, 1992). However, whether Rhombotin expression is restricted to intestinal endocrine cells as opposed to that of CRIP is currently unknown.

We have raised and characterised a number of LIM domain-specific peptide antisera against Rhombotin as well as Isl-1, and have compared the staining patterns of these antisera to those obtained with antisera specific for flanking epitopes. Our data from Western blotting and immunoprecipitation experiments clearly show that all antisera raised against synthetic peptides from both LIM domains of Rhombotin as well as from the distal LIM domain of Isl-1 indeed are highly specific towards these epitopes as they are presented in the corresponding recombinant proteins. In all cases, only the synthetic peptides used for immunisation were able to abolish the binding of the corresponding antiserum. It was therefore surprising that two antisera, raised against the same synthetic peptide (pep189, Figure 3B) from the first (N-terminal) LIM domain of Rhombotin, produced distinct staining patterns in the islet of Langerhans, i.e., Ab1976 stained α-cells on fixed and paraffin embedded sections and Ab1974 stained δ-cells on unfixed frozen sections (Figure 6 panels A–D *vs.* E–F). As in Western blotting the immunocytochemical staining reactions were highly specific as stainings were only abolished by preabsorption with the same synthetic peptide used for generating both antisera. Staining of islet sections with Abs 1974/1976 and Ab32 is expected not to distinguish between expression of Rhombotin and RBTN3, as the amino acid sequence of the immunising peptides are present in comparable positions in the two proteins (Figure 3B). The results from Figure 6 are taken to indicate that the N-terminal LIM-specific epitope, whether it is contained in Rhombotin or in RBTN3, is exposed differentially in the two cell types. We propose that differential α- and δ-cell specific exposure of the LIM-domain epitopes of Rhombotin/RBTN3 may reflect the presence of islet cell-type specific protein-protein interactions that have influence on the accessibility of the LIM-specific epitopes. This hypothesis is supported by the complete lack of reactivity of the LIM-specific anti-Rhombotin antibodies towards recombinant Rhombotin expressed in fibroblasts (cos-7 cells) which is otherwise readily recognised by immunocytochemistry on mono-layer cells through the N-terminal extension by the anti-FLAG antibody. Figure 7D furthermore shows that following lysis of the transfected fibroblasts the recombinant FLAG/Rhombotin could easily be immunoprecipitated with both Ab.1974 and 1976. It was additionally tested if any of the antisera would co-immunoprecipitate potential hetrodimerization partners but we found no consistency that could be correlated to a particular antibody specificity. Additionally, we tried to combine *in vitro* translated Rhombotin with nuclear extracts from cos-7 cells but could not reproduce masking of LIM-epitopes as observed in intact monolayer cells. Such masking should in theory compete with antibody recognition to diminish band intensity after immunoprecipitation. However, no such effects were detectable (Figure 7D) suggesting that the experimental conditions for immunoprecipitation is not compatible for retaining potential heterodimers with Rhombotin.

A similar phenomenon may explain the controversial staining patterns observed with Ab1958 raised against the second LIM-domain of Isl-1. Expression of Isl-1 in all four islet cell types has to date been shown by the use of two independently generated antisera raised against recombinant peptides, both enclosing the homeodomain and C-terminal region of Isl-1, expressed in *E. coli* either as a fusion protein (Dong *et al.*, 1991) or as a separate peptide fragment (Thor *et al.*, 1991; this study). Both these C-terminal specific antisera yield nuclear staining of all islet cell types, but in addition the antiserum raised by Dong *et al.*, 1991) also stains the cytoplasm of a subfraction of peripheral islet cells. In our study the LIM-specific Ab1958, selectively stained the cytoplasm of peripheral islet α-cells on rat islet sections (Figure 6 panel G and H), but no labelling of the nuclei was observed. As seen for Rhombotin the specificity of the two anti-Isl-1 antisera for the same protein on Western blots (Figure 5) was contrasted by the different staining patterns on islet sections (Figure 6G–J). This could indicate selective exposure in α-cell cytoplasm of the LIM-domain epitope which would otherwise be inaccessible to the antibodies in the nuclei of all islet cell types. As a corollary the Isl-1 epitopes recognised by the C-terminal-specific antiserum must be masked in the cytoplasm of the alpha-cell. Alternatively, proteolytic processing or alternative mRNA splicing separating the LIM domain from the homeodomain could explain a differential localisation of the two distinct epitopes in α-cells. However, only intact Isl-1 and not a LIM-only fragment of Isl-1 was detected on western blots containing extracts of insulinoma and glucagonoma tissue (O.D. Madsen, unpublished results). In addition, the nuclear localisation signal of Isl-1 is present N-terminally to the LIM domain which may indicate that this domain co-localises with the homeodomain to the nucleus.

Transient transfection of recombinant Isl-1 in cos-7 fibroblasts led to a strong staining of the nucleus in a large fraction of the cells in monolayer culture with the C-terminal-specific anti-Isl-1 antiserum. These cells showed marked differences in staining intensity which may reflect different levels of recombinant Isl-1 protein. Only a few cells of the transfected subpopulation displayed a nuclear staining with the LIM-specific antiserum indicating an efficient masking of the LIM domain epitope in the vast majority of the Isl-1 transfected cells. This subpopulation may represent the fraction of transfected cells with very high levels of recombinant Isl-1, which in turn may have titrated out a putative hetero-dimerisation partner. If correct, this dimerisation event is unlikely to be a prerequisite for nuclear localisation. Isl-1 was recently reported to be expressed in certain non-neuroendocrine cell lines, where its major site of location was cytosolic and not nuclear as in most endocrine cell lines (Wang & Drucker, 1994). This observation suggests the possibility that Isl-1 function might be regulated by sequestration in different cellular compartments. This is in support of our observation of cytosolic localisation of Isl-1-LIM specific

immunoreactivity in α-cells. Cytoplasmic localisation of LIM-only proteins has been preceded by the recently discovered Zyxin and the chicken cysteine rich protein (cCRP) which interact with each other at the focal adhesion site to the extracellular matrix (Sadler et al., 1992) through specific LIM-domain mediated hetero-dimerisation (Schmeichel & Beckerle, 1994).

In summary, we have cloned rat islet Rhombotin and demonstrated Rhombotin-like immunoreactivity in the somatostatin producing δ-cell and the glucagon producing α-cell. A series of antisera raised towards short sequences from the C-terminal loop structure of the proximal and distal Rhombotin LIM-domains as well as to the distal Isl-1 LIM-domain were all highly specific for the corresponding recombinant proteins as detected by Western blotting or by immunoprecipitation. Immunocytochemical application of these antisera predict a highly differential exposure of the LIM domain epitopes in different islet cell types. The data may suggest the presence of islet specific hetero-dimerisation partners interacting with the LIM domains in question or alternatively, that novel homologous cross-reacting LIM proteins are expressed in a cell type specific manner in the islet of Langerhans. The recent discovery that two other LIM-only proteins, RBTN2 and MLP, involved in erythropoiesis (Warren *et al.*, 1994) and myogenesis (Arber *et al.*, 1994), respectively, actually may mediate their function through modulations of the activity of cell specific helix–loop–helix proteins raise the possibility that the Isl-1 LIM-domain and Rhombotin may be modulators of helix–loop–helix proteins (Cordle *et al.*, 1991; German *et al.*, 1991) involved in islet specific gene expression.

Materials and methods

cDNA synthesis

cDNA was synthesised from 2 μg total RIN-5AH RNA in 20 μl reactions in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 25 μg oligo $(dT)_{12-18}$/ml (Pharmacia, Uppsala, Sweden), 100 mM DTT, 500 μM each of dATP, dGTP, dCTP and dTTP (Pharmacia) and 200 u M-MLV H-reverse transcriptase (Gibco, Gaithersburg, Maryland). Prior to addition of DTT and enzyme the RNA was heated to 70°C for 10 min and quick-chilled on ice.

PCR reactions

PCR reactions were performed in 100 or 50 μl reactions with 1 μl RIN-5AH cDNA as template in a buffer containing 10 mM Tris-HCl, 50 mM KCl. 1.5 mM $MgCl_2$, 0.01% gelatine, 0.1% Triton-X-100, 200 μM each of dATP, dGTP, dCTP and dTTP (Pharmacia), 1 μM each of the two primers and 2.5 U Taq-polymerase (Promega, Madison, Wisconsin). Amplifications were carried out by heating initially to 94°C for 2 min followed by 25 to 30 cycles with the following profile: 94°C 1 min, 60°C 2 min and 72°C 2 min, followed by a final extension step at 72°C for 10 min. Primers for cloning of rhombotin were: 5′-ATG.ATG.GTG.CTG.GAC.AAG.GAG-3′ (no. 168) and 5′-TTA.CTG.AAC.TTG.GGA.TTC.AAA.GGT-3′ (no. 170). The sequence occupied by the 5′ primer was verified by sequencing of a PCR product generated with primer no. 170 and a 5′ primer located upstream from exon 1a: 5′-GCC.AAA.GCG.CGC.CCC.GAG.TCG.G-3′ (no. 306). 3′-RACE used to confirm the sequence of the 3′ primer was performed by synthesising RIN-5AH cDNA with an adapter primer (RT-AP) (5′-GGC.CAC.GCG.TCG.ACT.AGT.AC-$(T)_{17}$-3′) instead of oligo(dT) as described above, and amplify the 3′ region of rat rhombotin cDNA by use of primer no. 168 and a primer specific for the adapter-primer sequence (AP) (5′-GGC.CAC.GCG.TCG.ACT.AGT.AC-3′), followed by re-amplification with a second gene specific primer internal to no. 168 (no. 816 5′GAA.GAA.CAA.CAT.GAT.CTT.GTG-3′).

Cloning of PCR products

The PCR product containing the entire rhombotin coding sequence was cloned directly into the pCR1000 vector (Invitrogen, San Diego, CA) according to manufacturers instructions. All constructs were verified by sequencing.

RT-PCR

Various organs (NEDH rats, Møllegaard Breeding Center, Denmark), isolated rat islets and phenotypically distinct islet tumor phenotypes (Madsen *et al.*, 1988; 1993a,b) were extracted for total RNA using guanidinium thiocyanate/phenol buffer (RNAzol, Cinna Biotex; Texas, USA) according to manufacturers recommendations. cDNA synthesis was performed as follows: 1 μg of total RNA was reverse transcribed at 37°c/1 h using 200 U M-MLV Reverse transcriptase (Gibco BRL), 40 U RNAsin (Promega), 3 μg random hexamers (Gibco BRL), 0.9 mM dNTP's, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT. PCR was performed for 25 thermal cycles (94°c/0′30″; 55°c/1′00″, 72°c/1′30″) using 1/25 of the synthesised cDNA under the following conditions: (all final conc.) 50 mM KCl, 10 mM Tris-HCl pH 9.0; 0.1% Triton X100, 1.5 mM $MgCl_2$, 20 μM dCTP, 40 μM dA,G,TTP's, 10 pmol of each primer, 2.5 U Taq Polymerase (Promega) and 2.5 μCi of 3000 Ci/mmol α-^{32}PdCTP as label. Products were analysed on a 6% acrylamide/7 M Urea standard sequencing gel. The sequence of the primers used are: Rhombotin upstream: TGC.CGA.TGC.TCT.CCG.TCC; downstream: GGT.TAG.CCT.TGG.TGT.AG, and G6PDH upstream: GAC.CTG.CAG.AGC.TCC.AAT.CAA.C; downstream: CAC.GAC.CCT.CAG.TAC.CAA.AGG.G.

In vitro *translation of cDNA*

In vitro translated full-length Rhombotin was synthesised from linearised pcDNA1-Neo-Rhombotin (inc. T7promoter) plasmid using the Riboprobe *in vitro* transcription kit (Promega), followed by translation using Rabbit Reticulocyte lysate (Promega) and ^{35}S-Methionine (Amersham, USA). All manipulations was performed according to manufacturers instructions. Nuclear extracts were prepared from cos-7 cells as described (Petersen *et al.*, 1994).

Direct sequencing of PCR products

PCR products were made with five-fold diluted primers, one of which was biotinylated at the 5′-end. DNA was prepared for di-dioxide sequencing by annealing the biotinylated amplification products contained in 40 μl of the PCR reaction to 200 μg streptavidin-conjugated paramagnetic beads (Dynal, Oslo, Norway) resuspended in 40 μl washing/annealing buffer (2 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) for 15 min at room temperature. The DNA/bead complexes were subsequently washed twice in washing/annealing buffer, followed by a single wash in TE-buffer. The two DNA strands were denatured with 10 μl 0.1 M NaOH for 5 min. The supernatant was transferred to 40 μl H_2O and DNA was recovered by ethanol precipitation. The bead-fraction was washed once with 0.1 M NaOH and once with TE-buffer. Sequencing was done with the T4 polymerase sequencing kit (Pharmacia) according to manufacturers instructions.

Expression of recombinant rhombotin and Isl1 in E. coli

The rat rhombotin coding sequence was subcloned into the single EcoRI site of the pAR (ΔRI)[59/60] vector, which tags an additional eight amino acids [the FLAG sequence (Blanar

& Rutter, 1992; Hopp *et al.*, 1988)] to the N-terminal part of the recombinant protein (Figure 3), and was transfected into bacteria of the BL21 *pLysS* strain (Studier *et al.*, 1990) obtained from Novagen (Madison, WI). After dilution of an overnight culture to an A_{600} of approx. 0.7 Rhombotin expression was induced by the addition of 1.5 mM IPTG for 3–4 h. Bacterial extract was made by sonication. The FLAG peptide sequence is recognised by a mouse monoclonal antibody, M2 (IBI, New Haven, CT), which makes it possible to trace expression of the recombinant protein independently of the LIM-specific epitope (Prickett *et al.*, 1989). Isl-1 was expressed from a related vector, pET3a lacking the FLAG sequences, and was kindly provided by Dr J. Leonard (Leonard *et al.*, 1992). Extracts from bacterial clones treated with IPTG to induce expression of the recombinant proteins were subjected to SDS–PAGE, and Western blotting.

Expression of recombinant Isl-1 and Rhombotin in Cos-7 cells

Monkey kidney fibroblasts (Cos-7) were cultured in DMEM, high glucose, supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (all from Gibco, Paisley, Scotland). The Isl-1 expression vector was as described (Leonard *et al.*, 1992). The Rhombotin coding region was amplified by PCR with 5′ primer (5′-GTG.TGC.GGA.TCC.AGG.TGC.CGC. CAT.-GGA.CTA.CAA.AGA.CGA.TGA.C-3′) containing a BamHI site followed by 12 bases corresponding to the sequence immediately 5′ to the start codon of the CRIP cDNA (Birkenmeier & Gordon, 1986) (serving as a translation consensus sequence), and a 3′ primer (5′ GCG.GGC.TTG.GAT.-CCG.CGG.CCG.CTT.ACT.GAA.CTT.GGG.ATT.CAA.-AGG.T-3′) containing a NotI restriction site. The FLAG/Rhombotin coding pAR(ΔRI)[59/60] construct was used as template. The amplification product was cloned into the expression vector pCDNA-1/Neo (Invitrogen), and the identity of the clone was verified by sequencing. Cos-7 cells were transiently transfected with the two constructs using the standard calcium phosphate precipitation procedure (Ausubel *et al.*, 1989).

Antibodies, peptides and immunocytochemistry

Peptide synthesis, immunisation, and immunocytochemistry were carried out as described elsewhere (Blume *et al.*, 1992). Peptide sequences are listed in Figure 3B. C-terminal Isl-1 antibodies were generated as described (Thor *et al.*, 1991) and anti-FLAG monoclonal antibody, M2, was from International Biotechnologies Inc., New Haven, CT. Monoclonal antibodies to insulin, glucagon (HUI-18 and GLU-001, Novo-Nordisk A/S, Bagsværd, Denmark) and the monoclonal rat antibody, CA812, (Contreas *et al.*, 1990) were used in two-colour immunofluorescence staining experiments on pancreatic sections to define islet β-, α-, and δ-cells, respectively, as described (Contreas *et al.*, 1990; Blume *et al.*, 1992).

Western blotting

Bacterial extract was run on 12–15% SDS gels in glycine buffer for 45–60 min at 40 mA/gel in a Mighty Small gel electrophoresis apparatus (Hoeffer, San Francisco, CA), and proteins were transferred to nitrocellulose filters by electroblotting. Filters were blocked by 1 h incubations in TBS with 3% BSA and in TBST with 3% dry milk powder. Filters were incubated o/n with anti-rhombotin antisera (diluted 1:500 in TBST/BSA with 15 mM NaN_3), with anti-Isl-1 antisera (Ab1958 diluted 1:1000; C-terminal Isl-1 antibody diluted 1:10000), and with the monoclonal anti-FLAG M2 antibody (0.1 μg/ml) in a wet chamber. Peptides for preabsorption were added in a concentration of 25 μg/ml. Alkaline phosphatase conjugated secondary antibodies ([goat anti-rabbit- (Zymed, San Francisco, CA) and rabbit anti-mouse (Dakopatts, Glostrup, Denmark) IgG for anti-rhombotin- and anti-FLAG antibodies, respectively) were diluted 1:1000 in TBST-milk and were incubated 2–4 h at room temperature. Blots were stained for 20–60 min with 0.3 mg/ml NBT and 0.2 mg/ml BCIP in 100 mM Tris pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$.

Immunoprecipitation

Rhombotin and Isl-1 transfected cos-7 cells were labelled by incubation for 4 h in [^{35}S]methionine-containing medium, and were extracted in hypotonic lysis buffer (10 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA). Undiluted antiserum (15 μl) was added to the protein extract (the equivalent of $10–30 \times 10^6$ c.p.m.) to a final volume of 150 μl and the samples were incubation o/n at 4°C. The samples were incubated with protein A sepharose for 30 min at 4°C, and washed five times with washing buffer (10 mM HEPES pH 7.4, 0.1 g/l BSA, 5 mM EDTA, 0.5% Triton X114, 10 mM benzamidine–HCl, 150 mM NaCl and 0.5 mM methionine). To characterise the immunoprecipitates the immune complexes bound to protein A sepharose were boiled for 3 min in 1 M Tris, 60% v/v sucrose, 15% w/v SDS, 0.01% w/v bromphenol blue, pH 6.8, with 1% β-mercaptoethanol, centrifuged and the supernatant subjected to 10% SDS–PAGE followed by fluorography.

Acknowledgements

The expert technical assistance of Erna E. Pedersen, Heidi I. Jensen, Tove Funder, and Ragna Jensen is greatly appreciated. The pAR(ΔRI) *E. coli* FLAG expression vector was a gift from Dr Michael A. Blanar. Dr Palle Serup is acknowledged for helpful discussions. KL and JSP were supported by fellowships from The Danish Research Academy and Juvenile Diabetes Foundation International. The project was in part supported by grants from the Danish Diabetes Association and The Danish National Science Foundation.

References

Arber, S., Halder, G. & Caroni, P. (1994). *Cell*, **79**, 221–231.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1989). *Current protocol in molecular biology*. Wiley Interscience: Boston, MA, USA.

Birkenmeier, E. & Gordon, J.I. (1986). *Proc. Natl. Acad. Sci. USA*, **83**, 2516–2520.

Blanar, M.A. & Rutter, W.J. (1992). *Science*, **256**, 1014–1018.

Blume, N., Petersen, J.S., Andersen, L.C., Kofod, H., Dyrberg, T., Michelsen, B.K., Serup, P. & Madsen, O.D. (1992). *Mol. Endocrinol.*, **6**, 299–307.

Boehm, T., Foroni, L., Kaneko, Y., Perutz, M.F. & Rabbitts, T.H. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 4367–4371.

Boehm, T., Foroni, L., Kennedy, M. & Rabbitts, T. H. (1990a). *Oncogene*, **5**, 1103–1105.

Boehm, T., Greenberg, J.M., Buluwela, L., Lavenir, I., Forster, A. & Rabbitts, T.H. (1990b). *EMBO*, **9**, 857–868.

Boehm, T., Spillantini, M.-G., Soforoniew, M.V., Surani, M.A. & Rabbitts, T.H. (1991). *Oncogene*, **6**, 695–703.

Bourgouin, C., Lundgren, S.E. & Thomas, J.B. (1992). *Neuron*, **9**, 549–561.

Cohen, B., McGuffin, M.L., Pfeifle, C., Segal, D. & Cohen, S.M. (1992). *Genes and Dev.*, **6**, 715–729.

Contreas, G., Jørgensen, J. & Madsen, O.D. (1990). *Pancreas*, **5**, 540–547.

Cordle, S.R., Henderson, E., Masuoka, H., Weil, P.A. & Stein, R. (1991). *Mol. Cell. Biol.*, **11**, 1734–1738.

Dong, J., Asa, S.L. & Drucker, D.J. (1991). *Mol. Endocrinol.*, **5**, 1633–1641.

Ericson, J., Thor, S., Edlund, T., Jassell, T.M. & Yamada, T. (1992). *Science*, **256**, 1555–1560.
Fisch, P., Boehm, T., Lavenir, I., Larson, T., Arno, J., Forster, A. & Rabbitts, T.H. (1992). *Oncogene*, **7**, 2389–2397.
Foroni, L., Boehm, T., White, L., Forster, A., Sherrington, P., Liao, X.B., Brannan, C.I., Jenkins, N.A., Copeland, N.G. & Rabbitts, T.H. (1992). *J. Mol. Biol.*, **226**, 747–761.
Freyd, G., Kim, S.K. & Horvitz, H.R. (1990). *Nature*, **344**, 876–879.
German, M.S., Blanar, M.A., Nelson, C., Moss, L.G. & Rutter, W.J. (1991). *Mol. Endocrinol.*, **5**, 292–299.
German, M.S., Wang, J., Chadwick, R.B. & Rutter, W.J. (1992). *Genes and Dev.*, **6**, 2165–2176.
Greenberg, J.M., Boehm, T., Sofroniew, M.V., Keynes, R.J., Barton, S.C., Norris, M.L., Surani, M.A., Spillantini, M.G. & Rabbitts, T.H. (1990). *Nature*, **344**, 158–160.
Hempe, J.M. & Cousins, R.J. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9671–9674.
Hempe, J.M. & Cousins, R.J. (1992). *J. Nutrition*, **122**, 89–95.
Hopp, T.P., Prickett, K.S., Price, V.L., Lippy, R.T., March, C.J., Cerretti, D.P., Urdal, D.L. & Conlon, P.J. (1988). *Biotechnology*, **6**, 1204–1210.
Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. & Edlund, T. (1990). *Nature*, **344**, 879–882.
Leonard, J., Serup, P., Gonzales, G., Edlund, T. & Montminy, M. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 6247–6251.
Li, P.M., Reichert, J., Freyd, G., Horvitz, H.R. & Walsh, C.T. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 9210–9213.
Madsen, O.D., Andersen, L.C., Michelsen, B., Owerbach, D., Larsson, L.-I., Lernmark, Å. & Steiner, D.F. (1988). *Proc. Natl. Acad. Sci. USA*, **85**, 6652–6656.
Madsen, O.D., Karlsen, C., Nielsen, E., Lund, K., Kofod, H., Welinder, B., Rehfeld, J.F., Larsson, L.-I., Steiner, D.F., Holst, J.J. & Michelsen, B.K. (1993a). *Endocrinology*, **133**, 2022–2030.
Madsen, O.D., Serup, P., Karlsen, C., Lund, K., Petersen, H.V., Blume, N., Andersen, F.G., Jensen, J. & Michelsen, B.K. (1993b). *Biochem. Soc. Trans.*, **21**, 142–146.
McGuire, E.A., Davis, A.R. & Korsmeyer, S.J. (1991). *Blood*, **77**, 599–606.
McGuire, E.A., Hockett, R.D., Pollock, K.M., Bartholdy, M.F., O'Brien, S.J. & Korsmeyer, S.J. (1989). *Mol. Cell. Biol.*, **9**, 2124–2132.
McGuire, E.A., Rintoul, C.E., Sclar, G.M. & Korsmeyer, S.J. (1992). *Mol. Cell. Biol.*, **12**, 4186–4196.
Michelsen, J.W., Schmeichel, K.L., Beckerle, M.C. & Winge, D.R. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 4404–4408.
Nielsen, E., Welinder, B.S. & Madsen, O.D. (1991). *Endocrinology*, **129**, 3147–3156.
Petersen, H., Serup, P., Leonard, J., Michelsen, B. & Madsen, O. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 10465–10469.
Prickett, K.S., Amberg, D.C. & Hopp, T.P. (1989). *BioTechniques*, **7**, 580–589.
Rabbitts, T.H. & Boehm, T. (1990). *Nature*, **346**, 418.
Royer-Pokora, B., Loos, U. & Ludwig, W.-D. (1991). *Oncogene*, **6**, 1887–1893.
Sadler, I., Crawford, A.W., Michelsen, J.W. & Beckerle, M.C. (1992). *J. Cell Biol.*, **119**, 1573–1587.
Sánchez-García, I., Osada, H., Forster, A. & Rabbitts, T.H. (1993). *EMBO*, **12**, 4243–4250.
Sanchez-Garcia, I. & Rabbitts, T. (1994). *Trends in Genetics*, **10**, 315–320.
Schmeichel, K.L. & Beckerle, M.C. (1994). *Cell*, **79**, 211–219.
Studier, F.W., Rosenberg, A.H., Dunn, J.J. & Dubendorff, J.W. (1990). *Meth. Enzymol.*, **185**, 60–89.
Thor, S., Ericson, J., Brännström, T. & Edlund, T. (1991). *Neuron*, **7**, 881–889.
Valge-Archer, V., Osada, H., Warren, A., Forster, A., Li, J., Baer, R. & Rabbitts, T. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 8617–8621.
Wang, M. & Drucker, D. (1994). *Endocrinology*, **134**, 1416–1422.
Warren, A., Colledge, W., Carlton, M., Evans, M., Smith, A. & Rabbitts, T. (1994). *Cell*, **78**, 45–57.
Way, J.C. & Chalfie, M. (1988). *Cell*, **54**, 5–16.
Xue, D., Finney, M., Ruvkun, G. & Chalfie, M. (1992). *EMBO*, **11**, 4969–4979.