Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation

Graham PACKHAM*†[1], Matthew BRIMMELL* and John L. CLEVELAND‡§

*Ludwig Institute for Cancer Research, Imperial College School of Medicine at St Mary's, London W2 1PG, U.K., †Virology and Cell Biology, Department of Medical Microbiology, Imperial College School of Medicine at St Mary's, London W2 1PG, U.K., ‡Department of Biochemistry, St Jude Children's Research Hospital, Memphis, TN 38105, U.S.A., and §Department of Biochemistry, University of Tennessee, Memphis, TN 38163, U.S.A.

The Bcl-2 oncoprotein is a key regulator of apoptosis and the Bag-1 protein interacts with Bcl-2 and cooperates with Bcl-2 to suppress apoptosis. The human *Bag*-1 cDNA is essentially identical with a previously described cDNA encoding RAP46, which interacts with activated steroid hormone receptors. However, there is considerable confusion over the structure of Bag-1/RAP46 proteins and their relationship to endogenous Bag-1 proteins. Here we have characterized Bag-1 expression in mammalian cells. We demonstrate that, in addition to the previously identified 32 kDa murine and 36 kDa human Bag-1 proteins, cells express a second 50 kDa Bag-1 isoform. In some murine cell lines p50 is expressed at the same level as p32 Bag-1, and p50 and p32 Bag-1 proteins have distinct subcellular localizations, suggesting that they are functionally distinct. The published mouse *Bag-1* cDNA is partial, and sequencing of additional murine *Bag-1* RNA 5′ sequences demonstrated that human and murine *Bag-1* cDNAs contain longer open reading frames than originally suspected. We determined which open reading frames gave rise to the Bag-1 isoforms in human cells. Suprisingly, translation of neither protein initiated at the first in-frame methionine, and cells do not express Bag-1/RAP46 proteins with the previously proposed structures; p50 Bag-1 initiates at an upstream CUG codon, whereas p36 Bag-1 initiates at a downstream AUG codon. Therefore, cells express two differently localized Bag-1 isoforms generated by alternative translation initiation, and Bag-1 proteins may play a dual role in regulating apoptosis and steroid hormone-dependent transcription.

INTRODUCTION

Bcl-2 family proteins are key regulators of apoptosis (programmed cell death) (for recent reviews see [1,2]). Protein–protein interactions are central to the function and regulation of these proteins and Bcl-2 forms homodimers and heterodimers with related family members, such as Bax. Bcl-2 has also been shown to interact with several non-family-member proteins, including NIP1, NIP2, NIP3, p53 BP2, Raf-1, CED-4, calcineurin, R-Ras and Bag-1 [1,2].

Murine Bag-1 contains 219 amino acid residues and has an apparent molecular mass of 29 kDa [3]. Although Bag-1 overexpression alone has little affect on rates of apoptosis of Jurkat T-cells, Bag-1 co-operates with Bcl-2 to suppress Fas-mediated and staurosporine-induced and cytotoxic T-lymphocyte-induced apoptosis [3]. More recently, Bag-1, like Bcl-2, has been demonstrated to interact with the Raf-1 serine/threonine kinase, although neither Bag-1 nor Bcl-2 is phosphorylated by Raf-1 [4,5]. In addition, Bag-1 has been identified as a hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) receptor binding protein, and Bag-1 enhances suppression of apoptosis by HGF or PDGF [6]. Thus, Bag-1 interacts with multiple cellular targets and suppresses apoptosis in several systems.

Recently, Takayama et al. [7] reported the isolation and sequence of a human *Bag-1* cDNA and identified a human Bag-1 protein with a molecular mass of 34 kDa. The human *Bag-1* cDNA is essentially identical with the previously reported *RAP46* cDNA, which was identified in a screen for proteins that interacted with the activated glucocorticoid receptor, and RAP46 protein was subsequently shown to interact with several activated steroid hormone receptors [8]. The *Bag-1/RAP46* cDNA has been proposed to contain a 274 amino acid open reading frame (ORF), beginning at the first in-frame methionine, that gives rise to human Bag-1/RAP46 proteins [7,8]; the 34 kDa human Bag-1 protein is therefore reported to contain an N-terminal extension of 55 amino acids relative to the murine protein [7]. However, there is considerable confusion and conflicting evidence regarding the relationship of this proposed ORF to Bag-1 proteins. Takayama et. al. [7] reported that, *in vitro*, the product obtained of the proposed ORF has a molecular mass of 34 kDa (Bag-1), whereas Zeiner and Gehring [8] reported a major product of 46 kDa (RAP46). More importantly, the relationship between the '*in vitro*' products of the *RAP46/Bag-1* cDNA and endogenous Bag-1 proteins has not been examined in detail.

In the present paper we have characterized *Bag-1* RNA and protein expression in mammalian cells and resolved these issues. In addition to the 32 kDa and 36 kDa Bag-1 isoforms previously identified in murine and human cells respectively, cells express a 50 kDa Bag-1 isoform. In murine myeloid cells, Bag-1 isoforms have distinct subcellular localizations, suggesting that they are functionally distinct. Finally, we demonstrate that cells do not express proteins with the structures previously assigned to either Bag-1 or RAP46 [7,8]; p50 Bag-1 translation initiates at a CUG codon upstream of the first in-frame methionine and p36 Bag-1 translation initiates at a downstream AUG codon. Thus, cells express two differently localized Bag-1 isoforms generated by alternative translation intiation.

Abbreviations used: DTT, dithiothreitol; FCS, fetal calf serum; GST, glutathione S-transferase; HGF, hepatocyte growth factor; NLS, nuclear localization signal; ORF, open reading frame; PDGF, platelet-derived growth factor; RACE, **r**apid **a**mplification of **c**DNA **e**nds; RPA, RNase protection assay; SV40, simian virus 40.

[1] To whom correspondence should be addressed

MATERIALS AND METHODS

Cell culture

WEHI-3 and NFS-56 murine myeloid cell lines, WEHI-22 and WEHI-279 murine T-cell lines and WEHI-231, NFS-70 and NFS-112 murine B-cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) [9,10]. The medium for WEHI-231 cells was additionally supplemented with 0.05 mM 2-mercaptoethanol. These cell lines were kindly provided by Dr. J. N. Ihle and Dr. D. S. Askew (St. Jude Children's Research Hospital, Memphis, TN, U.S.A.) or were obtained from American Type Tissue Culture (Rockville, MD, U.S.A.) The M1 mouse myeloid cell line, NIH3T3 fibroblasts and the SK-BR-7 and ZR-75-1 breast cancer cell lines [a gift from Dr. J. S. Burns and Dr. M. J. O'Hare (Ludwig Institute for Cancer Research, University College London, London, U.K.)] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS and antibiotics. The Epstein–Barr virus immortalized lymphoblastoid cell line X50-7 was maintained in RPMI-1640 supplemented with 10% (v/v) FCS.

Generation of anti-Bag-1 antibody

The murine Bag-1 coding sequence [3] was amplified by PCR using primers CGC GGA TCC ATG GCC AAG ACC GAG GAG AT and AAG GAA TTC TTC AGC CAG GGC CAA GTT TG and cDNA was generated using total RNA prepared from mouse liver. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into plasmid GEX2TK (Pharmacia) which had been digested with the same restriction enzymes to give GEX-mBag-1. GEX2TK or GEX-mBag-1 plasmids were transformed into BL21 bacteria and glutathione S-transferase (GST) non-fusion and GST–mBag-1 proteins respectively, induced with 0.3 mM isopropyl β-D-thiogalactosidase for 2 h and purified using glutathione–Sepharose-4B beads (Pharmacia), as previously described [11]. Three rabbits were repeatedly immunized with GST–mBag-1 and screened for reactivity against the 32 kDa Bag-1 protein in murine cells by Western blotting. All three rabbits responded to immunization and large-scale bleeds were taken from the rabbit that displayed the best reactivity.

Western and Northern blotting

For Western blotting (immunoblotting), cells were collected by centrifugation and resuspended in RIPA [0.15 M NaCl, 1% (v/v) Nonidet P40, 0.5% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 50 mM Tris/HCl (pH 8.0) and protease inhibitors]. After 5 min on ice, the lysates were clarified by centrifugation and supernatants were collected. The protein concentration of a sample of the lysate was determined using the Bio-Rad assay with BSA as a standard. The remaining lysate was diluted with an equal volume of 2 × protein sample buffer [100 mM Tris/HCl (pH 6.8)/4% (w/v) SDS/0.2% (w/v) Bromophenol Blue/20% (v/v) glycerol/200 mM dithiothreitol (DTT)] and proteins were denatured by heating at 95 °C for 5 min. Equivalent amounts of cell lysates were resolved on SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose. The filter was incubated in blocking buffer [5% (w/v) dried non-fat milk in PBS] to block non-specific protein-binding sites, and antibody incubations were in binding buffer [5% (w/v) dried non-fat milk in 150 mM NaCl/10 mM Tris/HCl, pH 8.0 (TS buffer)]. After each antibody incubation, the filters were washed twice in TS buffer and twice in TST buffer [TS buffer supplemented with 0.05% (v/v) Tween]. Bound immunocomplexes were detected by enhanced chemiluminescence (ECL; Amersham) and luminography.

Anti-Bag-1 was used at a 1:2000 dilution and the rabbit anti-Bag-1 antibody C-16 (Santa Cruz Biotechnology, Inc.) was used at a concentration of 0.5 μg/ml. The mouse Bcl-2-specific hamster monoclonal antibody 3F11 (Pharmingen) was used at a concentration of 5 μg/ml, followed by a rabbit anti-hamster IgG antibody (Sigma) at a concentration of 1 μg/ml. The human Bcl-2-specific mouse monoclonal antibody C124 (Dako) was used at a final concentration of 1 μg/ml. The F_1-β-ATPase-specific rabbit antibody was a gift from Dr W. Neupert (Ludwig-Maximilians-Universität, Munich, Germany). The final antibodies were horseradish peroxidase-conjugated donkey-(anti-rabbit) Ig or sheep-(anti-mouse) Ig (both Amersham) and were used at a dilution of 1:5000 to 1:10000.

Isolation of total RNA and Northern hybridization were performed as previously described [12]. The murine Bag-1 probe was the *Bam*HI–*Eco*RI insert from GEX–mBag-1.

5′ Rapid amplification of cDNA ends (RACE)

Poly(A)$^+$ RNA was isolated from murine myeloid BXH2-4S cells using oligo(dT) cellulose and 5′ RACE was performed using the 5′/3′ RACE kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, RNA was converted into cDNA using the *Bag-1*-specific primer S8951 (ACGCGTCGACCATC-TCCTCGGTCTTGG), tailed with terminal transferase and dATP, and products were amplified using *Pfu* DNA polymerase (Stratagene) and oligo(dT) anchor primer [GAC CAG GCG TAT CGA TGT CGA C$(T)_{16}$] and Bag-1 primer T1114 (ACG-CGTCCCTCGGTTTGGGTCGCC). The PCR products were digested with *Sal*I and cloned into pBluescript KS(−) (Stratagene) that had been digested with *Sal*I and *Eco*RV. The sequence of the cloned inserts was determined by double-stranded sequencing (Sequenase version 2.0; U.S. Biochemical Inc.). Sequencing was performed on both strands and using multiple RACE clones, except for the 5′ 80 nts which were present in a single clone (plasmid RACE.3). The novel mouse *Bag-1* RNA sequence appears in the GenBank Nucleotide Sequence Database under accession number AFO19889.

RNase protection assay (RPA)

Plasmid RACE.3 was digested with *Sal*I and the riboprobe generated using T7 RNA polymerase. RPA was performed using total RNA from M1 cells (10 μg), or yeast RNA (10 μg) as a control, and the RPA II kit (Ambion) according to the manufacturer's instructions. Briefly, RNA was precipitated with gel-purified riboprobe (50×10^3 Čherenkov c.p.m.), resuspended in hybridization solution (20 μl) and incubated overnight at 42 °C. Unprotected probe was removed by digestion using RNase A/T1 and protected fragments were resolved on a denaturing polyacrylamide gel.

Cloning of *Bag-1/RAP46* cDNA 5′ truncations and point mutants and *in vitro* translation

Plasmids for *in vitro* translation of different regions of the *Bag-1/RAP46* cDNA were generated by PCR and standard cloning techniques. For plasmid p42, which contains *Bag-1/RAP46* cDNA nts 42–1164 (*RAP46* cDNA co-ordinates; [8]), cDNA prepared from methyl mercury hydroxide-treated HaCaT poly(A)$^+$ RNA [a gift from Dr. P Farlie (Ludwig Institute for Cancer Research, St Mary's Branch, London, U.K.)] was amplified by PCR in the presence of 10% (v/v) dimethyl sulphoxide using primers T6243 (CCCAAGCTTTGGGCGGTCAACAA-GTGCGG) and T6244 (CCGGGATCCAGAGACGGCAGA-GCTGGTGG). For plasmid p276, which contains *Bag-1/RAP46* cDNA nts 276–1111, cDNA prepared from X50-7 total

RNA was amplified by PCR using primers T0458 (CCCAAGCTTGCGATGAAGAAGAAAACCCGGCG) and T0459 (CCGGATCCTGCTACACCTCACTCGGCCAG). The PCR products were digested with *Bam*HI and *Hin*dIII and cloned into pBluescript KS(−) (Stratagene) digested with the same enzymes. For plasmid p406 (nts 406–1164), p42 was used in a PCR reaction with primers V1432 (CCCAAGCTTGAGATGAATCGGAGCCAGGAGG) and T6244 and the PCR product was digested with *Bam*HI and *Hin*dIII and cloned into KS(−). For plasmid p289 (nts 289–1164), p42 was digested with *Fok*I, treated with Klenow polymerase and then digested with *Bam*HI and the cDNA sequences cloned into KS(−) was digested with *Eco*RV and *Bam*HI. For plasmid p445 (nts 445–1164), p42 was digested with *Sal*I and *Bam*HI, and the cDNA sequences cloned into KS(−) were digested with the same enzymes.

Two point mutants were generated to disrupt translation initiation at the CTG codon at *Bag-1/RAP46* cDNA nt 64 (C1) and the ATG codon at nt 409 (A2). In plasmid p42m, a stop codon was introduced immediately downstream of C1 in p42 by amplification of p42 using primers V3060 (CCCAAGCTTTGGGCGGTCAACAAGTGCGGGCCTGGCTTAGC) and T6244. In plasmid p406m, the ATG codon was altered to GTG by amplification of p42 using primer V2398 (CCCAAGCTTGAGGTGAATCGGAGCCAGGAGG) and T6244. In each case, the PCR products were digested with *Hin*dIII and *Bam*HI and cloned into KS(−) digested with the same enzymes. The sequence of the 5′ ends of all mutants was confirmed by double-stranded DNA sequencing.

In vitro translations were performed using the TnT-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]-methionine, according to the manufacturer's instructions. After 1 h at 30 °C, reactions were diluted with 4 vol. of 1 × protein sample buffer and heated at 95 °C for 5 min before analysis by SDS/PAGE and fluorography. For each construct, translations were performed with multiple, individual clones and these gave identical results.

Subcellular fractionation

M1 cells were washed twice with PBS and then resuspended in lysis buffer [0.2 M sucrose, 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1 mM DTT, 5 mM $MgCl_2$ and protease inhibitors] and disrupted by three cycles of freeze–thawing. The lysate was centifuged at 600 ***g*** at 4 °C for 10 min and the pellet was recovered as the nuclear fraction. The supernatant was then centrifuged at 10000 ***g*** at 4 °C for 30 min in a microfuge and the pellet was recovered as the heavy membrane fraction. Finally, the resultant supernatant was centrifuged at 100000 ***g*** at 4 °C for 60 min using an Optima TLX ultracentrifuge (Beckman) and the pellet was recovered as the light membrane fraction; the final supernatant was the cytosol. The pellets were washed with lysis buffer and then resuspended in lysis buffer equivalent to the volume of the cytosolic fraction. For the total extract, an equal number of unfractionated M1 cells were also resuspended in a volume of lysis buffer equivalent to the volume of the cytosolic fraction. Equivalent volumes of the fractions and total cell extract were diluted with an equal volume of 2 × protein sample buffer and heated at 95 °C for 5 min. The total and nuclear fractions were sonicated briefly to shear genomic DNA and the samples were analysed by Western blotting.

RESULTS

Mammalian cells express two Bag-1 isoforms

To analyse Bag-1 expression, we performed immunoblot analysis of lysates prepared from mouse NIH3T3 fibroblasts and M1 myeloid cells and the human lymphoblastoid cell line, X50-7, using two Bag-1-specific antibodies. Anti-Bag-1 is a polyclonal antibody raised against a chimaeric protein containing the mouse Bag-1 protein fused to the C-terminus of GST. C-16 recognizes an epitope within amino acid residues 204–219 of mouse Bag-1. This sequence is well conserved in human Bag-1 sequences [7]. The major Bag-1 proteins in human and mouse cells migrated with apparent molecular masses of approximately 36 and 32 kDa respectively, similar to the 34 kDa and 29 kDa molecular masses previously reported (Figure 1 in [7]). Anti-Bag-1 detected Bag-1 expression only in mouse cells and is therefore specific for murine Bag-1 proteins (Figure 1A). Importantly, both antibodies also detected a 50 kDa reactive protein in mouse cells and C-16 detected a 50 kDa protein in human cells. Since anti-Bag-1 and C-16 detect distinct epitopes, the p50 protein in human and mouse cells is closely related to the previously recognized Bag-1 proteins, and p50 is a novel Bag-1 isoform. We will refer to the prototypical Bag-1 proteins in murine and human cells as p32 and p36 Bag-1 respectively, and the novel isoform as p50 Bag-1. Anti-Bag-1 also detected a 35 kDa reactive protein in NIH3T3 fibroblasts, but this was not detected in other murine cell lines (Figure 2A and results not shown) and is likely to be due to cross-reaction, since this protein was not detected by C-16.

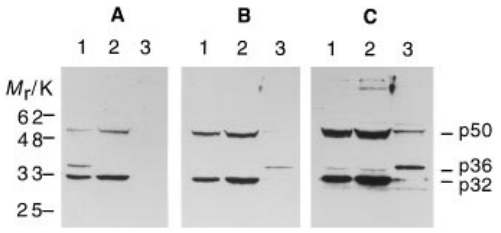

Figure 1 Mammalian cells express two Bag-1 isoforms

RIPA lysates (25 μg) prepared from murine NIH3T3 fibroblasts (lanes 1), M1 myeloid cells (lanes 2) and human X50-7 cells (lanes 3) were analysed by Western blotting using anti-Bag-1 (panel **A**) or C-16 antibodies (panels **B** and **C**). (**B**) and (**C**) are two exposures of the same Western blot. Positions of migration of the molecular-mass markers and the p50, p36 and p32 Bag-1 isoforms are indicated.

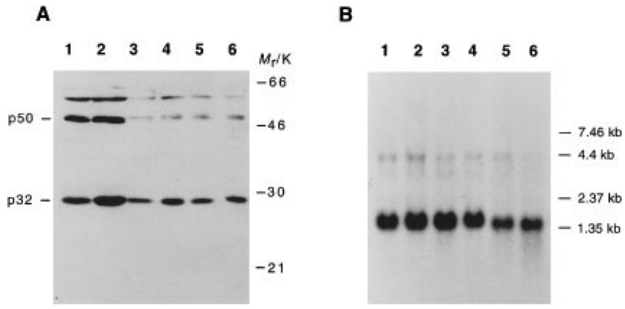

Figure 2 Expression of Bag-1 isoforms in murine haematopoietic cell lines

RIPA lysates (50 μg) or total RNA (10 μg) prepared from murine cell lines were analysed by (**A**) Western blotting using anti-Bag-1 or (**B**) Northern blotting using the murine *Bag-1* cDNA as a probe. Lane 1, WEHI-3; lane 2, WEHI-22; lane 3, WEHI-279; lane 4, WEHI-231; lane 5, NFS-70; lane 6, NFS-112. For the Western blot, the position of migration of Bag-1 isoforms and molecular-mass markers are shown. The slower-migrating protein observed in this experiment was not detected in other experiments (see for example Figure 1). The size of the major *Bag-1* RNA (1.4 kb) was determined by comparison with the migration of RNA ladder markers (Gibco). The Northern blot was also probed with a chicken β-actin cDNA probe to demonstrate equivalent loading in each lane (not shown).

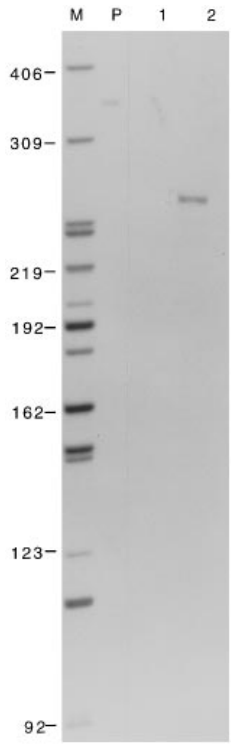

Figure 3 Analysis of mouse *Bag-1* RNA structure

A mouse Bag-1 5′ RACE clone containing 44 5′ bases of the published *Bag-1* cDNA and 203 bp of a sequence upstream of the start of this cDNA was used to generate a Bag-1-specific riboprobe. This was used as a probe in an RPA with M1 cell RNA (lane 2), yeast RNA (lane 1) was used as a negative control. Markers are ^{32}P-labelled *Msp*I digest of pBR322 DNA.

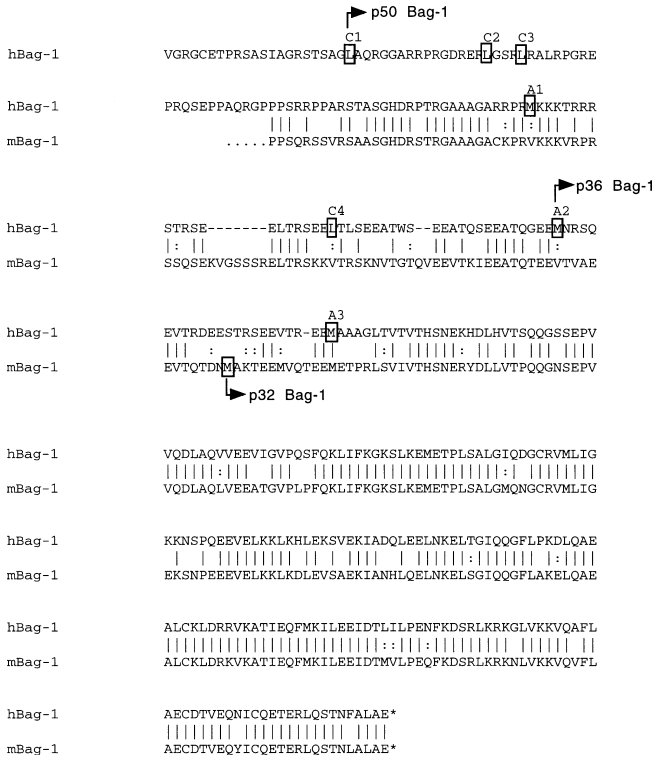

Figure 4 Human and mouse Bag-1 sequences

The alignment shows the hypothetical translations of the human *Bag-1/RAP46* cDNA [7,8] and the mouse *Bag-1* cDNA [3]. Identities are indicated by vertical marks and similarities by dots. The position of the potential translation initiation sites in the 5′ 500 bp of the human cDNA are boxed and termed C1–C4 and A1–A3 for in-frame CTG and ATG codons respectively. The first in-frame ATG (A1) has been proposed as the translation start site for the human p36 Bag-1 protein and the 46 kDa RAP46 protein [7,8]. However, our analysis demonstrated that p50 Bag-1 translation initiates at codon C1 and p36 Bag-1 translation initiates at codon A2.

Bag-1 RNA and protein expression in murine haematopoietic cell lines

To further characterize expression of Bag-1 isoforms, we used the anti-Bag-1 antibody to analyse murine haematopoietic cell lines. p32 Bag-1 was detected in all cell lines examined and levels were relatively constant (Figure 2A). In contrast, levels of p50 Bag-1 were more variable. p50 Bag-1 was highly expressed in some lines (WEHI-3, WEHI-22), at levels approximately equivalent to p32, whereas in other lines p32 was more abundant than p50. Finally, one cell line (NFS-56) had little or no detectable p50 expression (results not shown). The variable expression of p50 Bag-1 was not related to any particular cell lineage.

We next analysed *Bag-1* RNA expression in these cell lines by Northern blotting using the mouse *Bag-1* cDNA as a probe. A single major RNA was detected in all cell lines analysed, although some less abundant larger transcripts were also detected (Figure 2B). The modest variation in p32 Bag-1 expression, and the more significant variation in p50 Bag-1 expression, was not reflected in differences in *Bag-1* RNAs, and regulation of Bag-1 protein expression is therefore due to post-transcriptional mechanisms, at least in some cells. Notably, the major *Bag-1* RNA was approximately 1.4 kb, significantly larger than the published mouse *Bag-1* cDNA [3].

Analysis of mouse *Bag-1* RNA structure

The sequence of the human *Bag-1* cDNA has been reported recently [7]. This is very similar to the previously reported *RAP46* cDNA [8]. Both cDNAs are approximately 1.3 kb and the few nucleotide differences between them (15, of which 8 alter the protein sequence) are likely to be due to sequencing errors. In contrast, the mouse *Bag-1* cDNA is approximately 900 bp [3], significantly shorter than the human *Bag-1/RAP46* cDNA and the endogenous *Bag-1* RNA in murine cells (Figure 2B). Primer extension analyses demonstrated that the cDNA lacks 5′ sequences (results not shown), and we therefore attempted to clone and sequence additional mouse *Bag-1* cDNA sequences. Using 5′ RACE, we obtained 203 bp of a novel sequence upstream of the 5′ end of the published mouse *Bag-1* cDNA. To confirm the structure of the 5′ part of the *Bag-1* RNA in cells, we performed an RPA using the longest RACE clone as a probe. Full-length protection of the probe was evident, and therefore the cDNA sequences we have cloned are contiguous with the published cDNA and represent the major *Bag-1* RNA in cells (Figure 3). Moreover, the start of the published *Bag-1* cDNA does not represent a major RNA start in mouse liver, as we did not detect a protected fragment consistent with an RNA with this structure (results not shown); therefore the published mouse *Bag-1* cDNA [3] is a partial clone. Although we have cloned additional 5′ *Bag-1* RNA sequences, Northern blot and primer-extension analyses suggested that the we still have not identified the extreme 5′ end of the murine *Bag-1* RNA. However, attempts to clone additional 5′ sequences have failed, perhaps due to secondary structure in this part of the RNA.

We compared the hypothetical translations of the entire mouse

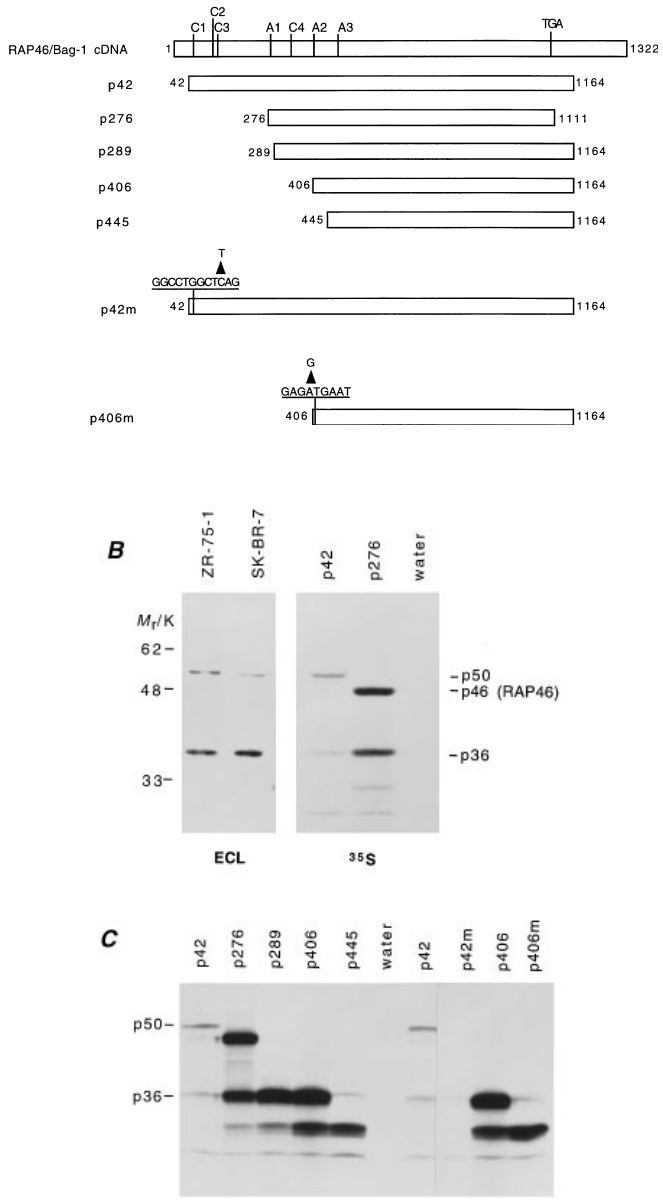

Figure 5 Identification of Bag-1 ORFs

(**A**) Structure of the constructs used to define the translation start sites of human Bag-1 isoforms. The position of the seven potential translation-initiation codons in the 5′ 500 bp of the *Bag-1/RAP46* cDNA (C1–C4 and A1–A3) and the stop codon are indicated. Below, the structure and nucleotide boundaries of each construct are indicated. The nucleotide alterations in plasmids p42m and p406m, which introduce a stop codon downstream of codon C1 and mutate codon A2 respectively, are shown. (**B**) RIPA lysates (25 μg) derived from two human breast cancer cell lines (SK-BR-7 and ZR-75-1) were analysed by Western blotting using the Bag-1-specific antibody C-16 (left panel, ECL). TnT lysates programmed in the presence of [^{35}S]methionine with plasmids p42 or p276, or water as a control, were analysed by autoradiography (right panel, ^{35}S). (**C**) TnT lysates programmed with the indicated plasmids in the presence of [^{35}S]methionine were analysed by autoradiography. The positions of migration of molecular-mass markers and Bag-1 isoforms are indicated.

and human *RAP46/Bag-1* cDNAs, including the sequence obtained from RACE (Figure 4). The first in-frame methionine in each sequence (termed A1 in the human sequence) has been proposed as the translation start site for mouse Bag-1 [3] and the 36 kDa human Bag-1 [7] and 46 kDa RAP46 [8] proteins. Suprisingly, identity between mouse and human sequences was also apparent upstream of the presumed initiating methionines, suggesing that these sequences are coding. Although this region of the human *Bag-1/RAP46* cDNA does not contain an in-frame ATG codon, it does contain three in-frame potential alternative CTG translation start codons (termed C1, C2 and C3; Figure 4). Although CUG-initiated translation is relatively rare, CUG-initiated forms of, for example, c-Myc, Int2 and Hck proteins have been described [13–15]. Thus, the human *Bag-1/RAP46* cDNA potentially contains extended ORFs which begin at CTG codons upstream of the first in-frame methionine proposed as the translation-initiation codon for p36 Bag-1 and RAP46 [7,8]. An extended ORF may also be present in the murine *Bag-1* RNA, as upstream sequences are also conserved with those present in the human.

Identification of human Bag-1 isoform translation start sites

There is considerable confusion in the literature concerning the structure of the products of the *RAP46/Bag-1* cDNA and their relationship to the proteins expressed in cells. For example, the product of the ORF starting at the first in-frame methionine has been described as a 46 kDa (RAP46) or a 36 kDa (Bag-1) protein [7,8]. Moreover, our analysis suggested that *Bag-1* cDNAs may contain longer ORFs than originally suspected. To resolve these issues we therefore determined which of the ORFs in the human *Bag-1* cDNA gave rise to the p50 and p36 Bag-1 proteins in human cells. Portions of the human *Bag-1/RAP46* cDNA were subcloned (Figure 5A) and these constructs were used to programme coupled transcription–translation reactions (TnT lysates) in the presence of [^{35}S]methionine; the protein products were analysed by autoradiography. The radioactive proteins were also compared with Bag-1 proteins in human cell lines detected using the C-16 antibody.

Translation of the largest Bag-1/RAP46 plasmid (p42, nts 42–1111) generated two proteins which comigrated with p50 Bag-1 and p36 Bag-1 expressed in human cells (Figure 5B). No proteins the size of RAP46 were detected in cell lysates, nor following translation of the full-length cDNA. In contrast, when sequences upstream of the first in-frame methionine were deleted in plasmid 276 (nts 276–1111), two major proteins of 46 and 36 kDa were generated. The size of the largest protein was consistent with that expected for RAP46, but a protein of this size was not detected in cells (Figure 5B). In contrast, the faster-migrating protein comigrated with p36 Bag-1 detected in human cells by immunoblotting (Figure 5B). Therefore, the *Bag-1/RAP46* cDNA encodes both of the Bag-1 isoforms, and sequences upstream of the first in-frame methionine are required for p50 Bag-1 translation. The faster-migrating proteins observed in these translations do not correspond to any Bag-1 proteins in cells, and are presumably due to artefactual internal intitiation. Introduction of a stop codon immediately downstream of codon C1 in plasmid 42m completely abolished translation of p50 Bag-1 but not p36 Bag-1 (Figure 5B). Therefore p50 Bag-1 translation initiates 5′ of the mutated codon, and since codon C1 is the only potential translation-initiation codon upstream of the introduced stop codon, p50 Bag-1 translation initiates at codon C1.

Since plasmids 42 and 276 directed translation of p36 Bag-1 *in vitro* (Figure 5B), p36 Bag-1 translation initiates downstream of codon A1. Three in-frame potential translation-inititation codons are downstream of A1, termed C4, A2 and A3 (Figure 4 and 5B). We therefore generated additional plasmids to determine which codon was used to initiate translation of p36 Bag-1. In lysates programmed with plasmids 289 and 406, which sequentially delete initiation codons A1 and C4 (Figure 5A), translation of

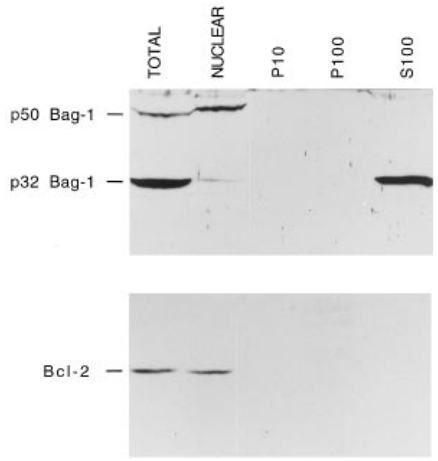

Figure 6 Distinct subcellular localizations of Bag-1 isoforms in M1 cells

M1 cells were fractionated by differential centrifugation and the resultant nuclear, heavy membrane (P10), light membrane (P100) and cytosolic fractions (S100) and a total cell extract derived from an equal number of cells were analysed by Western blotting using anti-Bag-1 (top panel) or anti-mouse Bcl-2 (bottom panel). The positions of migration of Bag-1 proteins and Bcl-2 are indicated. Analysis of the same fractions using an F_1-β-ATPase-specific antibody as a mitochondrial marker demonstrated that mitochondria were present in the nuclear fraction (results not shown).

the 46 kDa protein, but not p36 Bag-1, was abolished (Figure 5C). In contrast, when codon A2 was deleted in plasmid 445, p36 Bag-1 translation was abolished. Finally, mutation of codon A2 abolished p36 Bag-1 translation (Figure 5C). Therefore, p36 Bag-1 translation initiates at codon A2.

Bag-1 isoforms have distinct subcellular localizations in M1 cells

The 32 kDa Bag-1 isoform interacts with Bcl-2 [3], and since Bcl-2 associates with nuclear, mitochondrial and endoplasmic reticulum membranes [16–19] we determined the subcellular localization of Bag-1 isoforms in M1 murine myeloid cells fractionated by differential centrifugation. Immunoblot analysis of these fractions using anti-Bag-1 demonstrated that p32 and p50 Bag-1 have distinct subcellular localizations. p32 Bag-1 was detected almost exclusively in the cytosolic fraction (S100), whereas p50 Bag-1 was present in the nuclear fraction (Figure 6). This 'nuclear' fraction also contains mitochondria, as judged by detection of the mitochondrial marker F_1-β-ATPase in this fraction by immunoblotting (results not shown). Bcl-2 was detected exclusively in this fraction, consistent with the presence of nuclei and mitochondria. Since p32 Bag-1 was detected predominantly in the cytosol, this precludes the possibility that the nuclear fraction was contaminated with significant numbers of undisrupted cells. Therefore, Bag-1 isoforms localize differently within M1 myeloid cells, and p50 Bag-1, but not p32 Bag-1, co-fractionates with Bcl-2.

Recent experiments, carried out after the initial submission of the manuscript, demonstrate that N-terminal sequences present in human p50 Bag-1, but not p36 Bag-1, are sufficient to confer nuclear localization upon green fluorescent protein in transiently transfected Saos2 cells. Therefore p50 is likely to be a nuclear isoform (G. Packham and M. Brimmell, unpublished work)

DISCUSSION

In this report we demonstrate that murine and human cells express two Bag-1 isoforms. p50 expression is variable and in some murine cell lines p50 Bag-1 is expressed at levels equivalent to p32. Notably, Bag-1 isoforms also have different subcellular localizations in at least some cells, suggesting that p50 Bag-1 may be a functionally distinct form of Bag-1.

There has been considerable confusion in the literature concerning the structure and identity of Bag-1 and RAP46 proteins and we have resolved this confusion. Firstly, it is now clear that human *Bag-1* and *RAP46* are indeed the same gene, suggesting that, in addition to Bcl-2, Raf-1 and HGF and PDGF receptors [3,5,6], Bag-1 isoforms interact with activated steroid hormone receptors. Second, neither Bag-1 isoform is generated by translation initiation at the first in-frame methionine as previously proposed [7,8]. Rather, p50 Bag-1 translation initiates at an upstream CUG codon and p36 Bag-1 translation initiates at a downstream AUG codon. Therefore the major Bag-1 proteins in cells do not have the structures previously suggested by studies of either human Bag-1 or RAP46 [7,8]. Small amounts of the 46 kDa protein are detected in some cell lines that we have studied (results not shown), but we have never detected significant levels. Although sequences upstream of codon C1 lack an in-frame translation-termination codon, and it is possible that the cloned *Bag-1*/*RAP46* cDNAs do not represent the full extent of the RNA in cells, the cDNA contains all of the Bag-1 coding

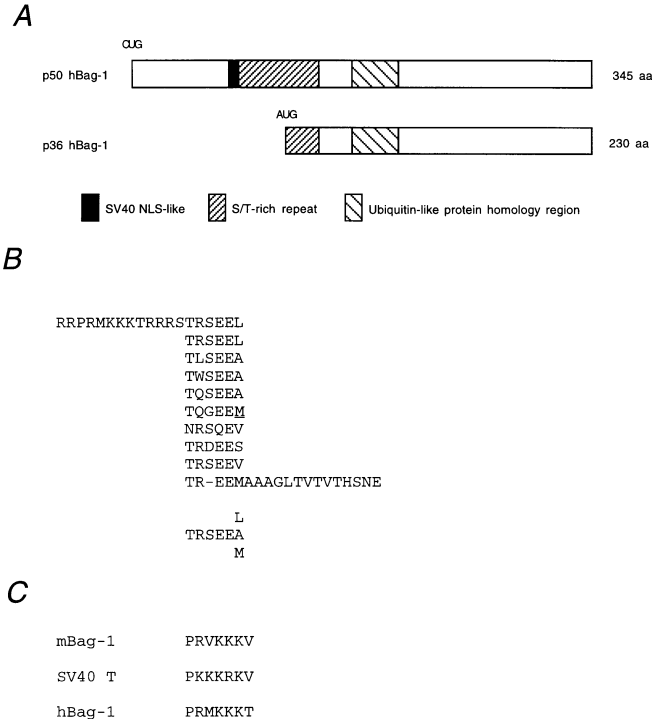

Figure 7 Summary of Bag-1 isoform structures

(**A**) The structures of the human Bag-1 proteins are shown in schematic form. NLS refers to the SV40 large T NLS-like sequence conserved in human and murine p50 isoforms (see **C**); S/T-rich repeat, serine/threonine-rich internal repeats (see **B**). (**B**) The human p50 Bag-1 internal repeat. Sequence alignment of the 11 copies of the repeat found in p50 Bag-1. The initiating methionine for p36 Bag-1 is underlined and a consensus is shown at the bottom. Similar sequences are present in murine Bag-1 proteins. (**C**) Simian virus 40 (SV40) large T NLS-like sequences in mouse and human p50 Bag-1.

information, since the largest product of the cDNA comigrates with the largest protein, p50 Bag-1, expressed in human cells.

Our analysis of Bag-1 isoform translation initiation has important implications for the domain structure of each isoform; the structures of the human Bag-1 proteins are shown diagramatically in Figure 7. The ubiquitin homology domain (p50 Bag-1 amino acid residues 169–200) first identified in murine p32 Bag-1 [3], is highly conserved in human Bag-1 proteins and, like the putative bipartite nuclear localization signal (NLS) identified in RAP46 [8], is present in both Bag-1 isoforms. However, in contrast to the ubiquitin-like domain, this putative bipartite NLS is not conserved in mouse Bag-1 proteins and therefore its significance is unclear. p32 Bag-1 is a soluble, cytoplasmic protein, whereas p50 Bag-1 is likely to be a nuclear protein. There are sequences in p50 Bag-1 similar to the simian virus 40 (SV40) large T NLS (Figure 7C), which, unlike the putative bipartite NLS [8], are conserved in mouse p50 Bag-1. Since this motif is partly encoded by cDNA sequences upstream of the first in-frame methionine, this putative NLS has not previously been recognized in Bag-1/RAP46 proteins.

The murine p32 and human p36 Bag-1 proteins are approximately 80% identical and 85% similar, and the p50 Bag-1 sequences, known for both human and mouse are approximately 75% identical and 80% similar (Figure 4). The region of lowest identity is in the central portion of the p50 molecules. This region of human p50 Bag-1 contains ten copies of a repeat sequence rich in serine, threonine and acidic amino acid residues (Figure 7B), which is predicted to adopt a largely helical secondary structure. p36 Bag-1 translation initiates towards the end of this region and contains only four copies of the repeat. Significantly, the equivalent portion of the mouse p50 Bag-1 protein contains 11 copies of a very similar repeat rich in serine, threonine and charged amino acid residues. Thus, although primary sequence similarity is modest, these parts of murine and human Bag-1 proteins may be functionally equivalent. Analysis of protein databases failed to reveal significant sequence similarity of this repeat with other proteins, although it is interesting that a steroid hormone receptor coactivator, SRC-1, also contains a region rich in serine and threonine amino acid residues [20].

Although relatively rare, translation initiation at CUG codons has been described for several proteins, including Hck, Int2 and c-Myc. Interestingly, similar to Bag-1 proteins, CUG- and AUG-initiated isoforms of Hck and Int2 are differently localized in cells [14,15], and the selection of translation-initiation codon may be used to control localization, and possibly activity, of Bag-1 proteins. *In vitro*, translation of p50 Bag-1 is inefficient, and deletion of the 5′ part of the cDNA greatly increases translation efficiency of the downstream ORFs. This is not due to a simple block in translation of the upstream ORFs, as blocking translation from codon C1 by introduction of a stop codon does not increase translation initiation at the downstream codons. Therefore, secondary structure in the 5′ end of the *Bag-1* RNA may prevent efficient translation of p50 Bag-1. This region has the potential to form stable stem–loop structures, and it is possible that regulation of *Bag-1* RNA structure also influences translation in cells.

Since *RAP46* and human *Bag-1* are the same gene, our results suggest that, in addition to interacting with Bcl-2, Raf-1 and HGF and PDGF receptors, Bag-1 isoforms bind activated steroid hormone receptors and may play a role in regulating hormone-dependent transcription as well as apoptosis. The *in vitro* binding between RAP46 and the oestrogen receptor is stable in 1 M urea [8], suggesting that binding to steroid hormone receptors is a high-affinity interaction and physiologically relevant. We are currently determining the activity of Bag-1 isoforms in regulating apoptosis and steroid hormone-dependent transcription, and whether Bag-1 proteins function independently, synergistically or antagonistically. In some cells, regulation of apoptosis and steroid hormone-dependent transcription are intimately linked (for example, dexamethasone-induced thymocyte apoptosis [21]), and Bag-1 proteins may be a direct functional link between these pathways.

This work was supported by the Ludwig Institute for Cancer Research (G.P.), Public Health Service grant DK44158 from the National Institutes of Health (J.L.C.), and the American Lebanese Syrian Associated Charities (ALSAC) (J.L.C.). We are very grateful to Dr Sheelagh Farrow for technical advice, Elsie White and Hui Yang for technical support, and Professor Paul J. Farrell and Drs Charles O. Rock, Xin Lu and Roger Watson for critical reading of the report. We thank Dr Walter Neupart for the F_1-β-ATPase-specific antibody, Dr Peter Farlie for the HaCaT cell cDNA, and Drs James Ihle, David Askew, Michael O'Hare and Jorge Burns for providing cell lines.

REFERENCES

1 Kroemer, G. (1997) Nature Med. **3**, 614–620
2 Reed, JC. (1997) Nature (London) **387**, 773–776
3 Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A. and Reed, J. C. (1995) Cell **80**, 279–284
4 Wang, H. G., Miyashita, T., Takayama, S., Sato, T., Torigoe, T., Krajewski, S., Tanaka, S., Hovey, L. E., Troppmair, J., Rapp, U. R. and Reed, J. C. (1994) Oncogene **9**, 2751–2756
5 Wang, H. G., Takayama, S., Rapp, U. R. and Reed, J. C. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 7063–7068
6 Bardella, A., Longati, P., Albero, D., Goruppi, S., Schneider, A., Ponzetta, C. and Comoglio, P. M. (1996) EMBO J. **15**, 6205–6212
7 Takayama, S., Kochel, K., Irie, S., Inazawa, J., Abe, T., Sato, Y. T., Druck, T., Huebner, K. and Reed, J. C. (1996) Genomics **35**, 494–498
8 Zeiner, M. and Gehring, U. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 11465–11469
9 Ihle, J. H. and Askew, D. S. (1989) Int. J. Cell Cloning **7**, 68–91
10 Pierce, J. H., DiFiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A. and Ihle, J. N. (1985) Cell, **41**, 685–693
11 Sinclair, A. J., Jacquemin, M. G., Brooks, L., Shanahan, F., Brimmel, M., Rowe, M. and Farrell, P. J. (1994) Virology **199**, 339–353
12 Cleveland, J. L., Dean, M., Rosenberg, N., Wang, J. Y. J. and Rapp, U. R. (1989) Mol. Cell. Biol. **9**, 5685–5695
13 Acland, P., Dixon, M., Peters, G. and Dickson, C. (1990) Nature (London) **343**, 662–665
14 Hann, S. R., King, M. W., Bentley, D. L., Anderson, C. W. and Eisenman, R. N. (1988) Cell **52**, 185–195
15 Lock, P., Ralph, S., Stanley, E., Bouler, I., Ramsey, R. and Dunn, A. R. (1991) Mol. Cell. Biol. **11**, 4363–4370
16 Akao, Y., Otsuki, Y., Kataoka, S., Ito, Y. and Tsujimoto, Y. (1994) Cancer Res. **54**, 2468–2471
17 Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W. and Reed, J. C. (1993) Cancer Res. **53**, 4701–4714
18 Lithgow, T., van Driel, R., Bertram, J. F. and Strasser, A. (1994) Cell Growth Differ. **5**, 411–417
19 Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J. and Shore, G. C. (1993) J. Biol. Chem. **268**, 25265–25268
20 Onate, S. A., Sophia, S. Y., Tsai, M.-J. and O'Malley, B. W. (1995) Science **270**, 1354–1357
21 Wyllie, A. H. (1980) Nature (London) **284**, 555–556

Received 4 July 1997/19 August 1997; accepted 21 August 1997