

Cell cycle-dependent association of Gag-Mil and hsp90

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Abstract

Immunoprecipitated p100Gag-Mil protein kinase from MH2-transformed quail embryo fibroblasts is associated with an 89 kDa protein. The molar ratio between p89 and Gag-Mil in the immunocomplex is 0.72, indicating that the majority of Gag-Mil is complexed with p89. During mitosis part of Gag-Mil is shifted to a form with reduced electrophoretic mobility, p102Gag-Mil. Appearance of p102Gag-Mil leads to a reduced association with p89 indicating that p102 is not associated with p89. Microsequencing of p89 isolated from immunoprecipitates of Gag-Mil identified the protein as the quail homologue of chicken hsp90. Our results show that p100Gag-Mil is associated with hsp90 with a high stoichiometry and that upshifted p102Gag-Mil is released from the complex with hsp90.

Key words: hsp90; Gag-Mil; c-Raf-1; Cell cycle; Microsequencing

1. Introduction

MH2 is an acutely transforming virus encoding two oncogene proteins: Gag-Mil and v-Myc [1]. Infection with the virus leads to carcinoma in chickens and both oncogenes contribute to the transforming potential of the virus [2]. The Mil portion of the Gag-Mil fusion protein is homologous to the carboxyterminus of c-Mil, the avian counterpart of the mammalian c-Raf-1 [3]. Gag-Mil is, like c-Raf-1, a serine-threonine specific protein kinase [4]. c-Raf-1 can be divided into a regulatory aminoterminal and a carboxyterminus with intrinsic kinase activity. c-raf is thought to gain transforming potential through loss of regulatory sequences encoded in the aminoterminal part of the protein rather than being activated through point mutations. Point mutations are rare and not conserved between different viruses carrying homologous sequences [5]. Indeed consecutive deletions in the aminoterminal half of the kinase enhance the transforming potential of the resulting proteins [6]. The minimal portion of the protein with the highest transforming potential is represented by v-Raf, the transforming principle of 3611, a murine leukemia virus. However, in the case of Gag-Mil, the Mil portion still contains part of the regulatory region of c-Mil [5].

Viral Mil/Raf proteins are implicated in various neoplasms [5] and although they have been under investiga-

tion for several years the molecular mechanisms of their action is still obscure. Lately a first *in vivo* substrate for the c-Raf-1 kinase has been discovered and shown to be associated with the carboxyterminus of c-Raf-1 [7]. Recent evidence [8] reveals a role for c-Raf-1 in meiosis of *Xenopus* oocytes and there is one report linking c-Raf-1 to later events in the cell cycle [9]. However, in the past, work has focussed on the activation of c-Raf-1 in G₁ following application of various mitogens [10]. c-Raf-1 is an essential intermediate between cell stimulation and proliferation [11]. c-Raf-1 activation leads to hyperphosphorylation and activation of the kinase while the viral Mil/Raf proteins are thought to be constitutively active. Major progress in linking cell stimulation and proliferation has been made by the discovery of physical interaction between activated Ras and c-Raf-1 [12].

In order to identify proteins associated with Mil/Raf proteins *in vivo*, we made use of MH2-transformed quail fibroblasts to co-immunoprecipitate associated proteins. In our study we show the isolation of one such protein with an *M_r* of 89 kDa and its identification as hsp90. Furthermore our data clearly demonstrate that the association between Gag-Mil and hsp90 is cell cycle-dependent.

2. Materials and methods

2.1. Cells and cell culture conditions

All cells were cultured at 37°C in a water-saturated atmosphere containing 7% CO₂. MH2-transformed quail fibroblasts [13] and MC29Q8NP [14], a non-virus-producing cell line derived from transfect-

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tion of quail fibroblasts with MC29 virus, were cultured in 47% DMEM/47% RPMI/5% FCS/1% chicken serum/0.5% DMSO supplemented with 50 mM HEPES and 1.5 g/l tryptose phosphate broth (DIFCO) containing penicillin (70 units/ml) and streptomycin 0.1 mg/ml. Rat1 cells and Rat1-derived cell lines were cultured in DMEM containing 10% FCS and antibiotics. To obtain cell lines that stably express the carboxyterminal half of hu-c-Raf, Rat1 cells were transfected with pMAMneoBLUEcRafCT (G. Radziwill, unpublished) and selected with 0.8 µg/ml G418. pMAMneoBLUEcRafCT allows Dexamethasone-inducible expression of hu-c-Raf-1 from amino acid 322 to 648 (c-RafCT). Clones were checked for expression of c-RafCT by immunoprecipitation of [³⁵S]methionine metabolically labelled cells with appropriate antibody and the clone Rat1RCT was chosen for further analysis. For metabolic labelling, exponentially growing cultures were washed twice with PBS and cultured for 3 h in methionine-free DMEM (ICN FLOW) containing 10% dialysed FCS and 150 µCi/ml Tran³⁵S-label (ICN FLOW). For synchronization of cells at different phases of the cell cycle exponentially growing cultures were treated for 17 h with Aphidicholine (G₁ phase) [15], arrested by double Thymidine block (S phase) [16], or treated for 10 h with 0.05 µg/ml Nocodazole (M phase) [17] prior to harvesting. To control for synchronization, 1 × 10⁵ cells were analyzed for DNA content after propidium iodide staining with a FACScan (Becton-Dickinson) using CellFIT software. All cell culture materials were obtained from Gibco.

2.2. Antibodies and immunoprecipitation

Antiserum directed against the carboxyterminal peptide CTTTSPQSPVF of hu-c-Raf-1 was obtained following immunization of rabbits with BSA-coupled peptide. Ascitic fluid of the previously described hybridoma producing Gag-specific antibody, [14] was prepared for immunoprecipitation. A Myc-specific rabbit serum was prepared using a MS2-Myc fusion protein as antigen [18]. For immunoprecipitation of interphase cells, cultures were washed twice with PBS and lysed in RIPA buffer (20 mM Tris pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 1 mM PMSF, 20 U/ml Trasylol (Bayer), 1 µg/ml leupeptin (Sigma), 1 µg/ml ribonuclease A, 50 mM 2-glycerolphosphate, 50 mM NaF, 1 mM NaVO₃) yielding 1 ml lysate per 1 × 10⁷ cells. For each lane 5 × 10⁶ cells were immunoprecipitated. The same buffer containing 50 mM NaCl, 0.5% Nonidet-P-40, 0.5% SDS without Triton X-100 was used for cell lysis under denaturing conditions. Nocodazole-treated cells were harvested by gently shaking the culture plates and pelleted at 800 × g prior to washing with PBS. Lysates were preadsorbed with 5% volume of protein A Sepharose (Pharmacia) for 30 min and spun down for 30 min at 11,000 × g. Supernatants were incubated for 2 h with 3 µl antiserum or ascitic fluid prebound to protein A Sepharose. In competition experiments this step was performed either in the presence of 10 µg/ml carboxyterminal Raf peptide or MS2-Myc protein. Immobilized immunocomplexes were washed 5 times with lysis buffer, SDS sample buffer was added and samples were incubated 3 min at 95°C prior to separation on 10% SDS polyacrylamide gels. For visualization of precipitated proteins, gels were either fluorographed with Kodak intensifying screens at -70°C using preflashed films or silver stained [19]. Gels with ³⁵S-labelled samples were quantified on a Phosphorimager (Molecular Dynamics), stained gels on a Personal Densitometer (Molecular Dynamics) using BSA as standard.

2.3. Protein sequence analysis

In order to obtain enough material for microsequencing, immunoprecipitation was scaled up. A total of 1 × 10⁹ MH2-transformed quail fibroblasts was immunoprecipitated with Gag-specific antibody, subjected to SDS-PAGE and p89 was eluted from Coomassie (Serva) stained gels [20]. The pooled eluates were TCA precipitated, separated again by gel electrophoresis and blotted to PVDF membrane (Millipore). p89 excised from Ponceau S (Sigma) stained membranes was cleaved by endoproteinase Lys C and processed for protein sequencing essentially as described [21]. In brief, unsaturated protein binding sites of the membrane were blocked with a solution containing 0.5% PVP-40 and 40% methanol, washed and digested in a buffer containing 100 mM Tris-HCl pH 8.5, 10% Acetonitril and 1% hydrogenated Triton X-100. 0.48 units of endoproteinase Lys C (Boehringer) were added to cleave 6 µg p89 bound to the membrane. After incubation for 16 h at 37°C resulting peptides were eluted from the membrane in digestion buffer with 0.1% TFA. The resulting peptides were separated on a µRPC

C2/C18 SC 2.1/10 (Pharmacia) column using the Smart TM System (Pharmacia). The column was run at a flow rate of 0.5 ml/min employing an aqueous gradient of Acetonitril in 0.1% TFA: 0–5 min, 0%; 5–60 min, 40%; 60–100 min, 80%; 100–105 min, 90%; 105–120 min, 90%. Sequence analysis of the indicated peptides was carried out using a pulsed liquid gas phase sequencer model 477A equipped with a model 120A PTH-aminoacid analyser (Applied Biosystems).

Computer aided searches and alignments were performed on a VAX/VMS 8600 system in the Protein Identification Resource databank release 25 (National Biomedical Research Foundation) and the Swiss-prot databank using FASTA programme [22] of GCG7 [23].

3. Results

3.1. Association of Mil/Raf proteins with p89

MH2-transformed quail embryo fibroblasts express the oncogene fusion protein p100Gag-Mil [14]. On analysis of immunoprecipitates of p100Gag-Mil with Mil/Raf-specific carboxyterminal peptide antibody several coprecipitating proteins from cells lysed under native conditions are detected in silver stained gels (Fig. 1A). Competition of the antibody with its cognate peptide antigen removes p100Gag-Mil and a protein with the *M_r* of 89 kDa from the immunoprecipitates. A second independently derived antibody directed against the amino-terminus of the viral p19Gag also precipitates both proteins. No coprecipitating proteins are observed with both antibodies under more stringent lysis conditions (Fig. 1A and data not shown).

To ensure that the observed coprecipitation is mediated by the carboxy terminus of p100Gag-Mil and not by the Gag moiety immunoprecipitation of another Gag-fusion protein was performed, p110Gag-Myc from MC29-transformed quail fibroblasts. It was immunoprecipitated in parallel to p100Gag-Mil under native lysis conditions with both, Myc- and Gag-specific antibodies. Prior to lysis cells were metabolically labelled with [³⁵S]methionine to allow more sensitive detection of coprecipitated proteins. Although a strong band of p110Gag-Myc is detectable in fluorographs no specifically coprecipitating 89 kDa protein could be visualised, while p100Gag-Mil-associated p89 was readily detectable (Fig. 1B).

In order to assess whether association with p89 is a conserved feature among Mil/Raf proteins a cell line (Rat1RCT) was established expressing the carboxyterminal half of human c-Raf-1 (c-RafCT). In the parental cell line (Rat1) the amount of c-Raf-1 is too low to allow detection of associated proteins in immunoprecipitates (Fig. 1C). In Rat1RCT expression level of c-RafCT is enhanced about sevenfold compared to c-Raf-1. Competition of the Raf-specific antiserum leads to the specific disappearance of c-Raf-1, c-RafCT (*M_r* = 38 kDa), several minor proteins with *M_r* between 39 and 29 kDa and a p89 from immunoprecipitates (Fig. 1C). In contrast to p89 the proteins with *M_r* between 39 and 29 kDa also appear in immunoprecipitates performed under denaturing lysis conditions identifying these as splice variants

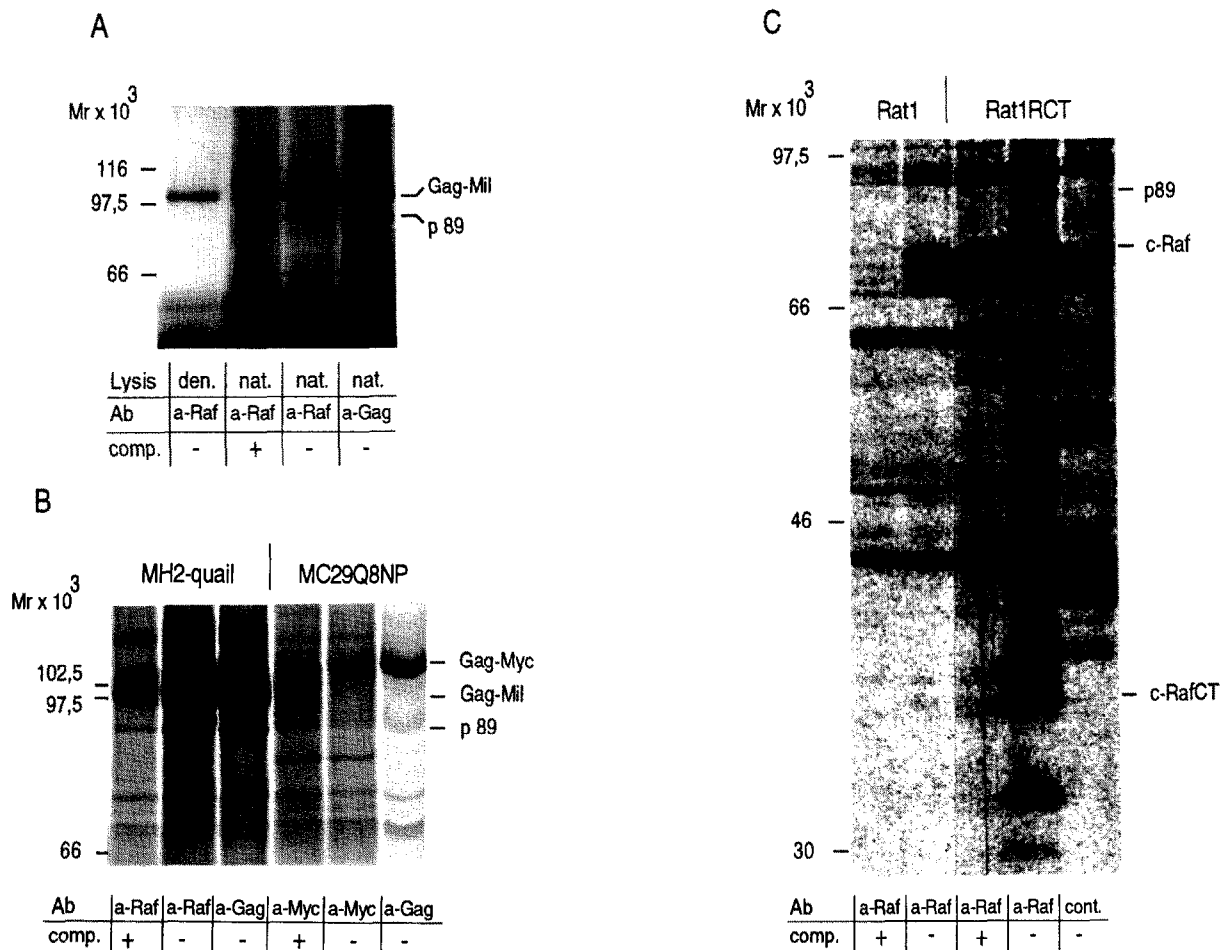


Fig. 1. Association of Gag-Mil/c-RafCT with p89. (A) MH2-transformed quail fibroblasts were immunoprecipitated under native (nat.) or denaturing (den.) lysis conditions using as antibody (Ab) Raf- specific peptide serum (a-Raf) or a monoclonal antibody against p19 Gag (a-Gag). Competition (comp.) was performed using the appropriate peptide in excess to saturate the antibody. The precipitates were separated by SDS-PAGE and silver stained as described in section 2. (B) Equal numbers of MH2-transformed quail fibroblasts or MC29Q8NP cells were labelled metabolically with [³⁵S]methionine, immunoprecipitated and separated on an SDS-polyacrylamide gel. Myc- specific antibody (a-Myc) was competed with excess of MS2-Myc fusion protein. A fluorograph of the dried gel is shown. (C) Same as in (B), except that Rat1 and a Rat1 derived cell line (Rat1RCT) stably transfected with the carboxyterminal moiety of c-Raf-1 (c-RafCT) were immunoprecipitated either with Raf-specific or unrelated control antibody (cont.). Cells were stimulated with 1×10^{-7} M Dexamethasone at the beginning of metabolic labelling.

or aminoterminal degradation products of c-RafCT, while p89 is absent from immunoprecipitates under such conditions (data not shown). p89 is also absent from immunoprecipitates with unrelated control antibody (Fig. 1C).

3.2. Cell cycle-dependent association of Gag-Mil and p89

Although the action of c-Raf-1 is well established during G₁-phase there is evidence suggesting a role for Mil/Raf proteins in later phases of the cell cycle [9]. Therefore we investigated the association of Gag-Mil and p89 in populations of cells enriched at different stages of the cell cycle. Cells synchronized at G₁- or S-phase showed no obvious differences compared to unsynchronized cells in immunoprecipitates of Gag-Mil (data not shown). However, after enrichment of cells in M phase of the cell cycle a different relative mobility of Gag-Mil as well as a low-

ered stoichiometry of association between Gag-Mil and p89 were observed (Fig. 2A and C). Irrespective of the protocol used for synchronization, either transiently by means of release from double thymidine block in S-phase (data not shown) or stationary by means of Nocodazole block in M-phase (Fig. 2A), a more slowly migrating form of Gag-Mil with an *M_r* of 102 kDa appeared (Fig. 2A). p102 was detected by means of silver staining in immunoprecipitates of both Gag-Mil-specific antibodies. Appearance of p102 was also observed in immunoprecipitates obtained under denaturing lysis conditions and in Western blot analysis of whole cell lysate (data not shown). In both cases presence of p102 was abolished by competing the antibody with its cognate antigen. p102 and p100Gag-Mil derived from [³²P]orthophosphate metabolically labelled cells show identical phosphopeptides in two-dimensional peptide maps

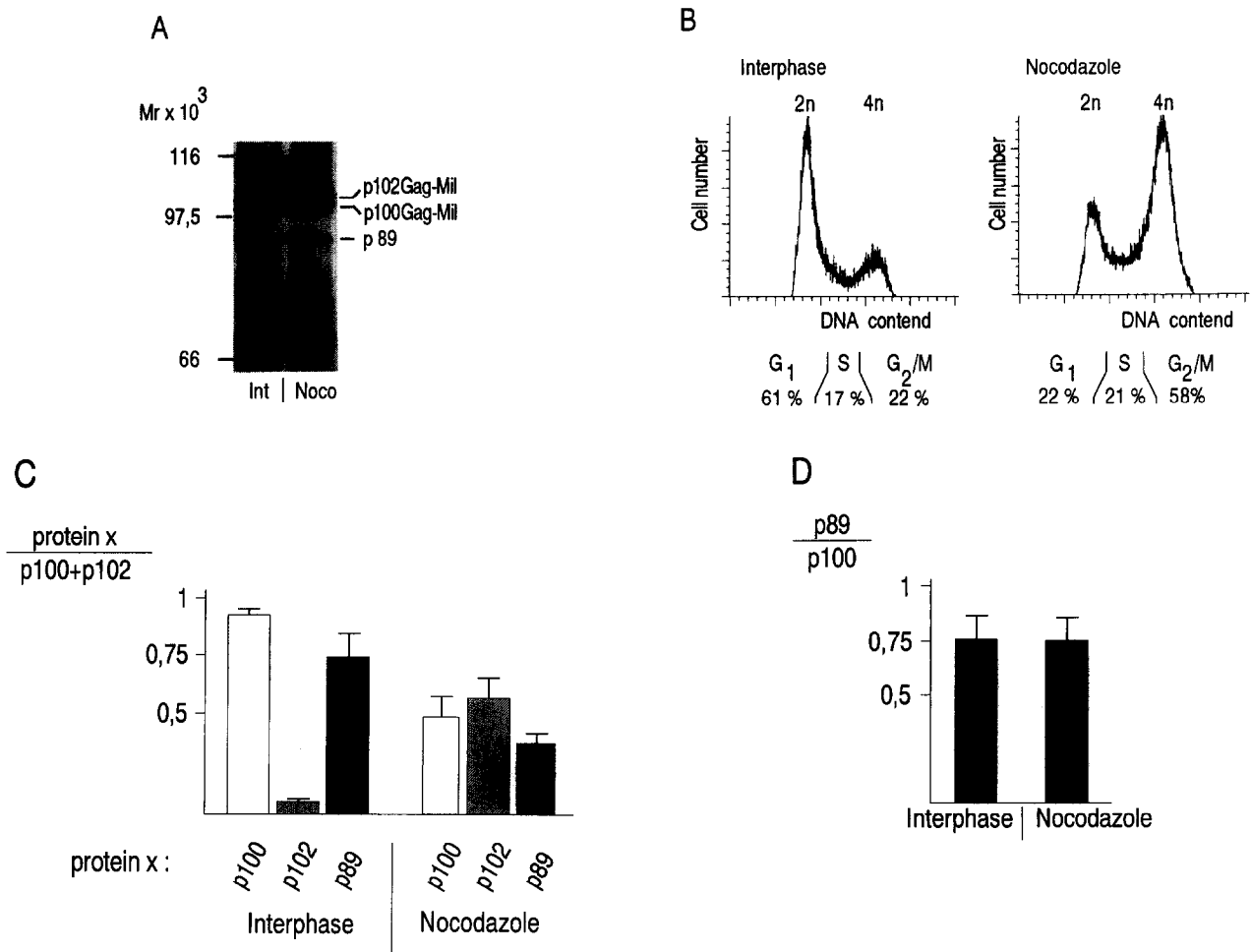


Fig. 2. Association of Gag-Mil and p89 is cell cycle-dependent. (A) Exponentially growing interphase (Int) or Nocodazole-treated (Noco) MH2-transformed quail fibroblasts were immunoprecipitated under native conditions as in Fig. 1A. Positions of p89, p100Gag-Mil and the mitosis-specific p102Gag-Mil are indicated on the right. (B) FACS-analysis of the DNA content from MH2-transformed quail fibroblasts cultured in parallel with the cells used in (A). 2n and 4n indicate the ploidy of the cells. (C and D) Quantification of four independent assays similar to the one shown in (A). Results are shown as means $\pm$ standard errors of four independent assays. Indicated are the molar ratios of p100Gag-Mil (open boxes), p102Gag-Mil (hatched boxes), or p89 (black boxes) to total Gag-Mil (p100Gag-Mil in addition to p102Gag-Mil) present in the immunoprecipitates. Boxes in D indicate molar ratios of p89 to p100Gag-Mil present in immunoprecipitates.

(manuscript in preparation). From these properties of p102 we conclude that it is indeed a modified form of p100Gag-Mil and name it therefore p102Gag-Mil.

In interphase cells where almost all of Gag-Mil is present as p100, p89 and Gag-Mil are associated in a molar ratio of 0.72. In cells enriched for mitosis the molar ratio of p89 versus total Gag-Mil (p100 and p102) in the immunoprecipitates is lowered to 0.35 (Fig. 2C). This means that association is lowered to 48% of the value observed in interphase cells. At the same time the fraction of Gag-Mil present as p100 is lowered from 93% in interphase cells to 46% in Nocodazole-treated cells. Therefore, if one calculates the molar ratio between p89 and p100Gag-Mil present in immunocomplexes the value is identical between interphase cells and cells enriched in mitosis (Fig. 2D), indicating that the mitosis-specific p102Gag-Mil is not associated with p89.

3.3. Identification of p89 as hsp90

To further characterize the 89 kDa coprecipitating protein we took advantage of the fact that it was present in relatively high amounts in immunoprecipitates of p100Gag-Mil. About 40 ng of p89 were isolated from immunoprecipitates of 5×10^6 cells (Fig. 1A). Furthermore, no contaminations by other cellular proteins with the same M_r as p89 were detectable after competition of the antibody (Fig. 1A). In order to obtain enough material to allow microsequencing of the protein, immunoprecipitation was scaled up. p89 was eluted from gels loaded with immunoprecipitates from lysates corresponding to 10^9 MH2-transformed quail fibroblasts. The amount of p89 recovered was about 11 μ g in total. Because of aminoterminal blockage towards Edman degradation (data not shown), part of the protein (6 μ g) was digested by endoproteinase Lys C. The resulting peptides

were separated by Microbore HPLC chromatography and two of them were sequenced on an automated sequencer (Fig. 3A). Computer-aided search for homology to the obtained sequences was carried out. A 100% match to chicken and human hsp90 was found for the 20 amino acid stretch present on the sequenced peptides (Fig. 3B). We therefore conclude that the 89 kDa protein associated with Gag-Mil in a cell cycle-specific manner is the quail homologue of chicken hsp90.

4. Discussion

Association of Mil/Raf proteins with other proteins was observed at two points of Mil/Raf action; in the course of kinase activation [12] and during substrate phosphorylation [7]. In order to isolate new Mil/Raf associated factors we used a rather direct approach trying

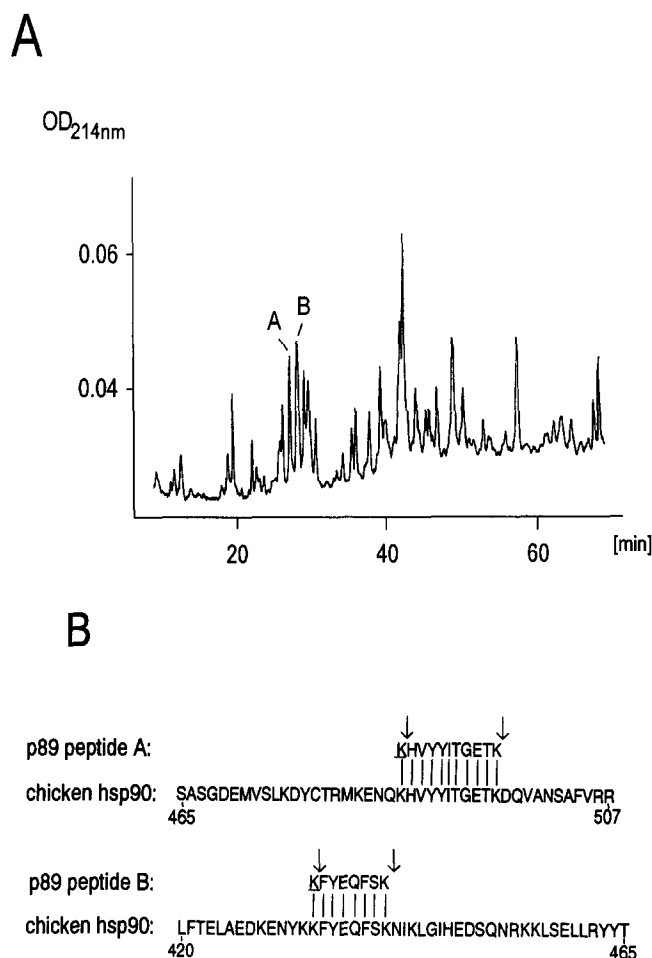


Fig. 3. Protein sequence analysis of p89. (A) Peptide map of p89 generated by digestion with endoproteinase Lys C and separation by HPLC. The peptides A and B were used for aminoterminal sequence determination. (B) The obtained sequences of peptide A and B were aligned to the amino acid sequence of chicken hsp90. The identity of underlined residues was deduced from the sequence specificity of Lys C, indicated by arrows.

to coimmunoprecipitate these proteins. To obtain enough material to characterize associated proteins we employed the naturally occurring system of Gag-Mil overexpression in MH2-transformed quail fibroblasts. These cells express p100Gag-Mil in an amount up to 0.015% of total cellular protein (J. Lovrić, unpublished observation). In this study we describe the isolation of p89, the most abundant coprecipitating protein in Gag-Mil immunoprecipitates. Furthermore we show that its association with Gag-Mil is cell cycle-dependent and identify p89 as hsp90.

Co-immunoprecipitation of p89 and p100Gag-Mil is observed with both aminoterminal and carboxyterminal antibodies and is abolished by competing the antibody or by more stringent lysis conditions (Fig. 1A), indicating that p89 is a specifically associated protein rather than a degradation product of p100Gag-Mil. In MC29Q8NP the Mil-specific antibody did not precipitate relevant amounts of proteins with a M_r of 89 (data not shown) and precipitates of p110Gag-Myc also do not show significant amounts of such proteins (Fig. 1B). The lower amount of immunoprecipitable p110Gag-Myc compared to p100Gag-Mil is in part due to the strong DNA binding of Myc [25]. However, under conditions which release Gag-Myc from DNA, no association between Gag-Mil and p89 is detectable (Fig. 1A, first lane). c-RafCT, which is homologous to part of the Mil sequences in p100Gag-Mil, also associates with a p89 in Rat1 cells. Taken together these data represent strong evidence for an association of p89 and the catalytic domain of Mil/Raf proteins in vivo. The detection of the associated proteins by silver staining allows a reliable estimate of the ratio of p89 versus p100Gag-Mil in immunoprecipitates. Aminoterminal and carboxyterminal antibodies result in the same high stoichiometry of association, rendering it unlikely that the antibodies prefer binding to p89-associated Gag-Mil rather than to unbound Gag-Mil. If one assumes a 1:1 ratio of Gag-Mil and p89 in the complex and the possibility that part of the p89 is released from the complex during immunoprecipitation, then most if not all of the p100Gag-Mil present in interphase cells is associated to p89. Note that the stoichiometry of association between p89 and p100Gag-Mil detectable in metabolically labelled cells (Fig. 1B) compared to direct staining of immunocomplexes (Fig. 1A) is lowered from 0.72 to about 0.15. This is probably caused by differences in protein synthesis rates during the labelling time of 3 h and differences of in vivo protein half-lives between p89 and Gag-Mil. An increase of stoichiometry to about 0.3 is achieved after metabolically labelling of cells with [³⁵S]methionine for 10 h (data not shown).

In mitotic cells the appearance of p102Gag-Mil correlates with a lower stoichiometry of association to p89. The portion of Gag-Mil appearing as p102 varied from 40% up to 65% of the total Gag-Mil in four independent

assays (Fig. 2C). These changes correlate with changes in molar ratios of p89 versus total Gag-Mil between 0.4 and 0.32 compared to 0.72 in interphase cells. No changes occur if one estimates the ratio of p89 versus p100Gag-Mil in immunoprecipitates of either mitotic or interphase cells (Fig. 3D). These results can be explained most easily by assuming that only p100Gag-Mil is associated with p89 and not the modified p102Gag-Mil.

Longer exposure of cells to Nocodazole leads to higher enrichment of mitotic cells with blocked mitotic spindles [17] and might therefore enhance the observed changes but it also lowers yields of Gag-Mil in immunoprecipitations (data not shown). This is probably due to degradation of Gag-Mil during prolonged M phase and the 10 h exposure to Nocodazole is a compromise in order to obtain enough mitotic cells with only modestly prolonging the M phase which has a duration of 1–2 h in an undisturbed cell cycle. During mitosis, cells round up and detach from the substratum. To rule out the possibility that the observed effects are simply due to loss of contact to the substratum cells were incubated in PBS without Mg^{2+} and Ca^{2+} . This treatment also leads to cells detached from the substratum. However, in these cells no changes in electrophoretic mobility of Gag-Mil or in association to p89 occurred (data not shown).

The sequenced peptides of p89 show 100% identity to chicken- and human hsp90 and p89 is therefore concluded to be the quail homologue of chicken hsp90 (Fig. 3B). Because of the relatively high abundance of hsp90 one might assume that it is present rather unspecifically in the immunoprecipitates and accumulated during isolation of p89. However, there are no contaminating proteins in the region of p89 migration on SDS polyacrylamide gels after competition of the antibody (Fig. 1A). Because of the low amount of protein used for sequencing, remaining contaminations from background proteins with identical electrophoretic mobility could not have led to any readable sequence.

While this work was under way L.F. Stancato and coworkers [26] showed the association of carboxyterminal Raf sequences with hsp90 by means of coimmunoprecipitation followed by hsp90-specific Western blot analysis. Our results support these findings and extend them in important aspects, namely by quantifying the molar ratios of the components and by demonstrating the high stability of the complex. Furthermore, we show that association of hsp90 and Gag-Mil is cell cycle-dependent. Further work is under way in order to investigate association of other Raf proteins and hsp90 during the cell cycle by means of coimmunoprecipitation followed by hsp90-specific Western blot.

hsp90 has been shown to associate with steroid hormone receptors and pp60^{v-src}. In the case of pp60^{v-src} hsp90 is only transiently associated with the inactive kinase and has been implicated in cytosolic trafficking while the active, tyrosine phosphorylated and mem-

brane anchored pp60^{v-src} is not associated with hsp90. However, transformation-deficient mutants of pp60^{v-src} are stably associated with hsp90 [27]. Furthermore, it is well established that pp60^{c-src} is activated during mitosis [28]. Our results indicate that there might be some parallels in kinase activation and in association to hsp90 between pp60^{src} and Mil/Raf proteins. Activation of c-Raf-1 kinase activity leads to hyperphosphorylation and decreased mobility of the kinase in SDS polyacrylamide gels [29]. A decreased mobility of c-Raf-1 in SDS gels is also observed in mitotic cells ([9] and manuscript in preparation). We also observe a decreased mobility of Gag-Mil in mitotic cells. In parallel to c-Raf-1, this mobility shift presumably results from an activation of the kinase and is accompanied by release of Gag-Mil from hsp90 association, as in the case of pp60^{v-src}. Furthermore it holds true for both proteins that their cellular counterparts c-Raf-1 and pp60^{c-src} are not observed in stable association with hsp90 without being overexpressed [26,30]. This could reflect either intrinsic differences between the oncogene products and their cellular counterparts or be a result of the low abundance of pp60^{c-src} and c-Raf-1. Further investigation of c-Raf-1 kinase activity and association with hsp90 will clearly be helpful in answering the question whether hsp90 plays a more general role in kinase activation and/or cytosolic trafficking of kinases.

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