p53 and the ribosomal protein L5 participate in high molecular mass complex formation with protein kinase CK2 in murine teratocarcinoma cell line F9 after serum stimulation and cisplatin treatment

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Abstract Using the murine teratocarcinoma cell line F9 we investigated the influence of serum stimulation and cisplatin treatment on the p53, CK2, MDM2 levels. Both treatments led to an increase of p53, though with different kinetics; the other proteins investigated were not affected. We present direct evidence by immunoprecipitation for an association of protein kinase CK2 holoenzyme ($\alpha_2\beta_2$), p53, and the ribosomal protein L5. The results suggest complexes between the CK2 holoenzyme and p53 but also p53/CKβ complexes. Furthermore we provide evidence for the existence of high molecular mass complexes of CK2 in vivo. This is the first evidence that, under physiological conditions, protein kinase CK2 does not exist solely as a heterotetramer, but predominantly in association with other proteins.

Key words: Protein kinase CK2; p53; L5; MDM2; Cisplatin

1. Introduction

Protein-protein interaction studies are becoming more and more the focus of general interest. Especially since the introduction of the yeast two-hybrid system almost a decade ago [1] the number of potential interacting proteins has increased considerably. Protein kinase CK2 is no exception. Recently a number of interacting proteins were identified [2,3], among them A-Raf [4,5], ribosomal protein L5 [2,6] and others. The yeast two-hybrid results suggesting an interaction between CK2β and A-Raf were confirmed in the baculovirus system showing a stimulatory effect on A-Raf kinase inasmuch as MEK phosphorylation was severalfold increased [4,5].

Here we present new evidence for an in vivo association of CK2 subunits with ribosomal protein L5 in non-overexpressing cells. Interestingly, L5 has been found complexed with MDM2 and MDM2/p53 complexes [7]. Since CK2 can phosphorylate p53 [8] and associate with p53 [9–11], a possible higher order complex formation including CK2/L5/p53 and MDM2 is suggestive. Moreover, it was shown that MDM2 is a substrate for CK2 and that p53 can enhance this phosphorylation [12].

Our present aim was to follow a strategy where a mammalian cell line – overproducing neither protein kinase CK2 subunits α and β nor the interacting partners under investigation, i.e. p53, protein L5 – was used throughout the experiments.

It was shown previously that the level of p53, after treatment of cells with serum or a mitogen, increased markedly (for review see [13]). However, a large body of evidence suggests that stabilization of p53 protein is the main mechanism to increase its levels. Thus stabilization occurs as a result of a decrease in degradation that increases the half-life of p53. One possibility to increase levels of p53 protein is by applying DNA-damaging agents, e.g cisplatin [14–16].

By applying a semiquantitative immunodetection method to follow the amounts of p53, L5, MDM2 and CK2 over a time course of 4 h we discovered that p53, after treatment of the cells with cisplatin, increased about 10-fold in less than 2 h after the insult.

2. Materials and methods

2.1. Cell culture

The mouse embryonal teratocarcinoma cell line F9 was obtained from the American Type Culture Collection (ATCC). Cells were grown on tissue culture plates treated with 0.1% gelatin at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Subconfluent cultures (cell density about 70–80%) were used throughout the experiments. The cells were treated with 0.01 mg/ml *cis*-diamminedichloroplatinum(II) (cisplatin) for 4 h or as indicated in the figure legends.

2.2. Preparation of crude cell extracts and chromatographic analysis

Cells were rinsed three times with phosphate buffered saline (PBS) and then resuspended in buffer containing 25 mM Tris-HCl pH 8.5, 1 mM DTT, 100 mM NaCl and a cocktail of inhibitors (Boehringer Mannheim). Disruption of the cells was carried out by sonication followed by removal of cellular debris with a $4000\times g$ centrifugation step for 20 min at 4°C.

The supernatant was immediately used for immunoprecipitation or for chromatographic analysis. Samples assigned for gel filtration were previously equilibrated in buffers containing 25 mM Tris-HCl pH 8.5, 150 mM NaCl or 1 M NaCl before loading onto a Smart System Superose 6 (Pharmacia) column.

2.3. Antibodies

Monoclonal antibody PAb 200.47 was kindly provided by Dr. C. Goetz (University of Saarland, Homburg/Saar, Germany). Purified anti-monoclonal p53 antibody (Ab-1) and anti-CK2α and anti-CK2β antibodies were obtained from Calbiochem while anti-polyclonal biotinylated p53 antibody was purchased from Boehringer Mannheim. Anti-monoclonal MDM2 antibody (SMP14) was obtained from Santa Cruz. For the immunoprecipitation polyclonal antibodies from rabbits directed against the human recombinant CK2 subunits or against a peptide of human L5 protein were used.

2.4. Immunoprecipitation

Cell extracts (400 μg) were diluted in IP buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.05% NP40, 0.02% NaN_3, 0.2% BSA, cocktail of inhibitors) and incubated with monoclonal or polyclonal antibodies overnight at 4°C in a total volume of 200 μl. The complexes were precipitated by incubation with protein A-agarose (Boehringer Mannheim) for 4 h at 4°C. Immunoprecipitates were washed five times with IP buffer and once with PBS and then sub-

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jected to protein kinase assays or analyzed on SDS-polyacrylamide gel.

2.5. Protein kinase assay

After treatment with serum or cisplatin for the times indicated in the figure legends, different amounts (1, 2.5, 5 μg) of F9 crude cell extracts were assayed at 37°C for 5 min using the synthetic peptide as a substrate (RRRDDDSDDD) [17,18]. 1 U of CK2 activity is defined as the amount of activity necessary to transfer (1 nmol phosphate/min)·10^{-1} into the synthetic peptide substrate at 37°C.

2.6. SDS-polyacrylamide gel electrophoresis and Western blot analysis

SDS-PAGE was carried out as previously described [12]. For the immunodetection of our proteins equal amounts of proteins were applied to the gel, based on results from protein determination of the cell extracts. However, in order to ensure maximum comparability, a so-called match gel was run first. The proteins were Coomassie stained and according to visual evaluation, if necessary, matched. If matching was successful, the gel for blotting followed. One blot was always used for the detection of all proteins of interest. This guaranteed that all proteins had undergone the same treatment and that transfer and washing conditions were always applied to all proteins in the same way. This was achieved by cutting the blot horizontally into several strips using the rainbow markers located on both sites to identify the position of the individual proteins, e.g. MDM2 (ca. 90 kDa), p53 (ca. 53 kDa), CK2α (ca. 44 kDa), L5 (ca. 34 kDa) and CK2β (ca. 26 kDa). Needless to say, before this protocol was applied, each antibody was tested on the whole blot in order to make sure that there were no unspecific cross-reactions. Any possible technical alterations would affect all proteins equally. The transfer from the gel to the membrane was also monitored by staining the membrane with Ponceau red and by staining the gel after the transfer with Coomassie blue.

3. Results

3.1. Short-time serum stimulation of F9 cells

In order to study the effect of short-time serum stimulation (4 h) on the expression of the CK2 subunits α and β, p53 and MDM2, F9 cells were starved for 48 h and stimulated with fresh medium containing 10% FCS. Cells were harvested at the times indicated and CK2 activity was determined. As one can see from the CK2 activity distribution no significant changes were observed (Fig. 1A). The individual proteins were also detected by immunostaining using specific antibodies. Equal amounts of protein were loaded on a gel. The immunoblots of the α and β subunits (Fig. 1A) did not show significant differences in signal intensity over the time period investigated. Hence, the results obtained are in agreement with the observed unchanged CK2 activity before and after serum stimulation (Fig. 1A). The same is true for the

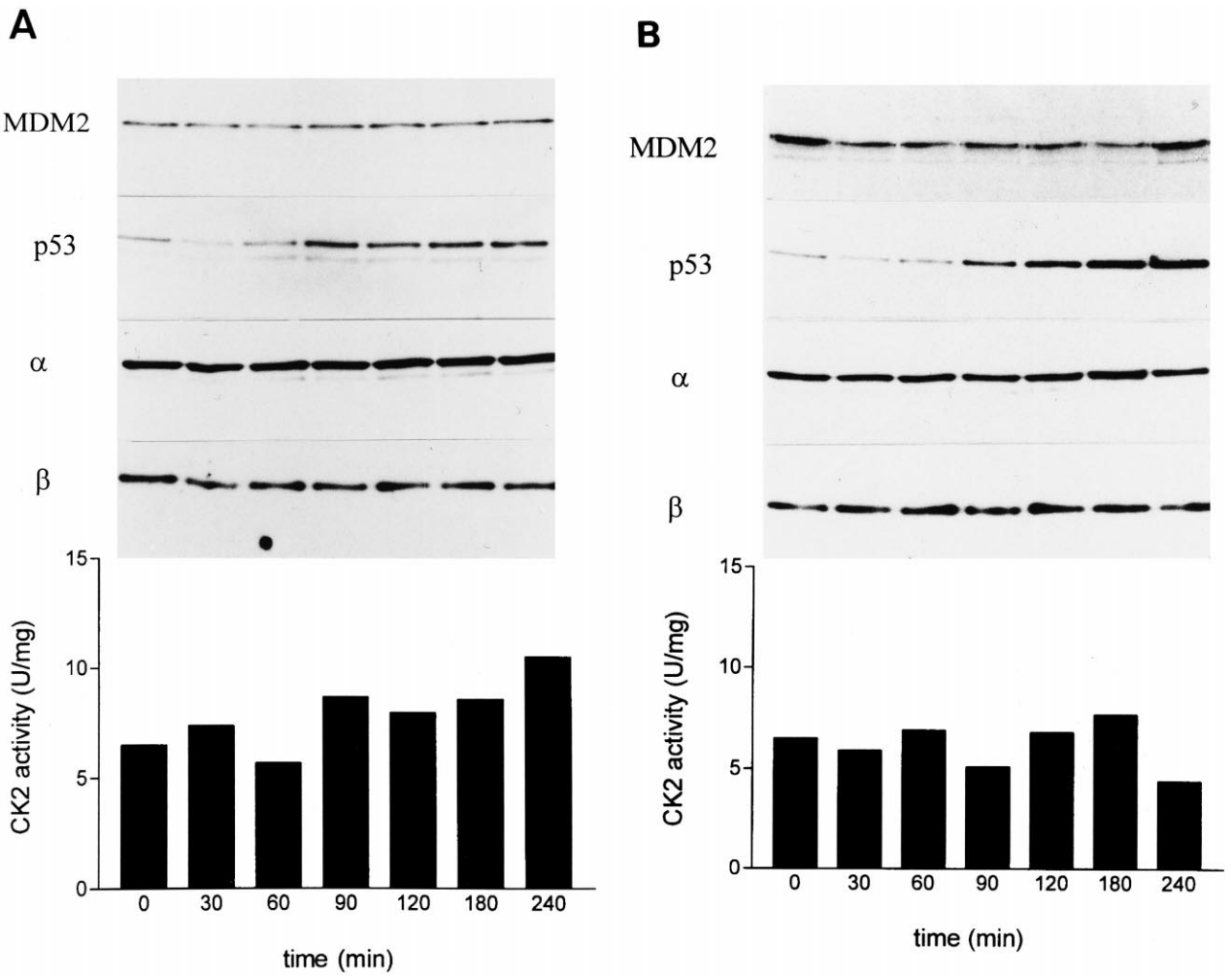

Fig. 1. A: Immunoblots showing the detection of proteins MDM2, p53, CK2α and β subunits together with the protein kinase CK2 activity (lower graph) over a time range from t=0 until 4 h after serum stimulation. Prior to serum stimulation the F9 cells were starved for 2 days by growth on DMEM in the absence of fetal calf serum. B: Same as in A, except that the cells were grown on DMEM, 10% FCS for 2 days after seeding before the addition of cisplatin.

MDM2 protein. Here too, we did not see any alterations in protein expression. A completely different picture arose from the time course of p53 where we observed a weak but distinct signal in the starved cells ($t = 0$). 30 min after serum stimulation the amount of p53 dropped by more than 50%, to a very faint band. But 90 min after serum stimulation the quantity of p53 was about 3–4-fold above the amount found in starved cells and it remained constant over the rest of the stimulation period.

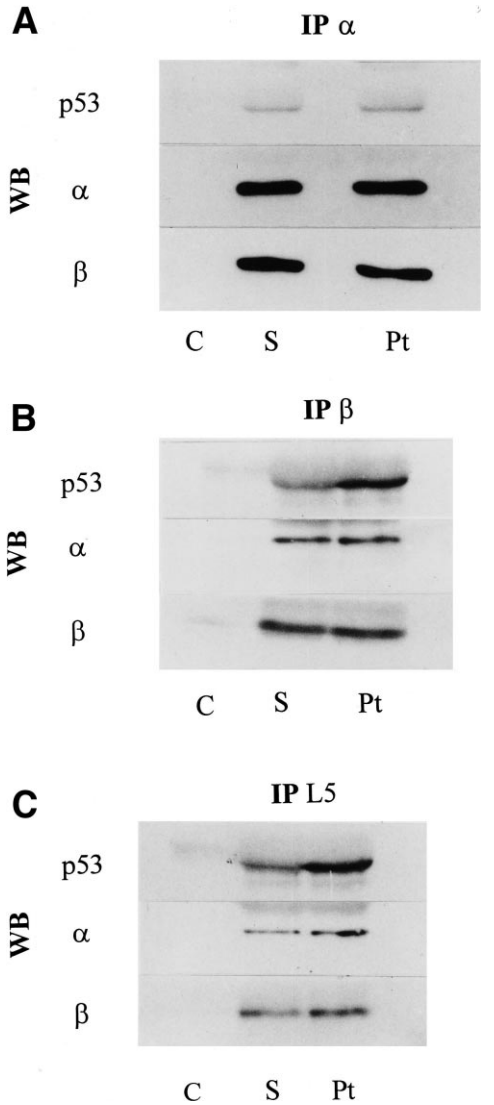

Fig. 2. A: Immunoblots from immunoprecipitation experiments (IPs) using a polyclonal rabbit antibody directed against the human CK2α subunit. The preimmune serum of the rabbit from which the antibody was obtained served as a control (C). 400 μg of a F9 cell extract was used for each IP. The extracts derived from cells which had been serum-stimulated (S) or which had been subject to cisplatin treatment (Pt). After IP the material was analyzed by SDS-PAGE and subsequently transferred to a membrane by Western blotting. The proteins of interest were visualized using an anti-p53 antibody (polyclonal biotinylated) and by using the monoclonal antibodies directed against CK2α (1AD9) and CK2β (6D5). B: Immunoblots from IPs using a polyclonal rabbit antibody directed against the human CK2β subunit. Otherwise everthing was the same as in A. C: Immunoblots from IPs using a polyclonal rabbit antibody directed against the human ribosomal protein L5. Otherwise everthing was the same as in A.

3.2. Treatment of F9 cells with cisplatin

Next, we wanted to answer the question whether treatment of the cells with cisplatin (DNA-damaging agent leading to stabilization of p53) would have a similar effect. We observed that as in the case of serum stimulation CK2 activity was unaffected. Also the amount of the CK2 subunits α and β and of the oncogene product MDM2 was unchanged over the 4 h drug treatment. Again, in the case of p53 we found a background level of p53 before cisplatin was added. After 30 min of cisplatin treatment we observed a decline of the p53 level. However, 60 min later the p53 expression level was back to that found before treatment with the drug. After 90 min the amount of p53 had already risen above the level found in starved cells. A doubling of p53 in comparison to the background level was observed 90 min after drug treatment. However, 4 h after drug treatment there was still an increase noticeable in contrast to the p53 alterations observed after serum stimulation where the maximum was reached after 90 min (Fig. 1B).

3.3. Immunoprecipitation with anti-CK2α

In order to answer the question whether we can demonstrate mutual association of protein L5, CK2, and p53, immunoprecipitation experiments were conducted in mammalian cells that do not overexpress these proteins under investigation. First we used a polyclonal antibody directed against CK2α (IPα). The precipitated complexes were analyzed on SDS-PAGE followed by Western blotting and immunostaining using monoclonal antibodies directed against CK2α (WBα), CK2β (WBβ) and p53 (WBp53). We can see that both CK2 subunits are detected, supporting the notion that the holoenzyme was precipitated with the anti-CK2α antibody (Fig. 2A). The three membrane strips were incubated for immunodetection for equal times. This should not only allow the qualitative detection but also give a semi-quantitative estimate of the relative distribution of the three proteins in the extracts. Despite the difference in avidity of the antibodies used for the detection of the CK2 subunits (1AD9 and 6D5) we could establish – using CK2 holoenzyme as an internal standard for a 1:1 subunit detection – a suitable dilution ratio permitting the detection of imbalances in the CK2 subunit ratio in cell extracts. There is no difference in the amount of p53 from serum-stimulated cells (S) and those that had undergone cisplatin treatment (Pt). C is the control where pre-immune serum was used for the immunoprecipitation.

3.4. Immunoprecipitation with anti-CK2β

Fig. 2B shows the results obtained when anti-CK2β was used for the IP. The first lane is again the control, where a pre-immune serum was used for the immunoprecipitation. The second lane shows the results when serum-stimulated cell extracts were used and the third lane shows cisplatin-treated cell extracts after immunoprecipitation.

Clearly, in the case of CK2α and β the result obtained is as expected. The antibody directed against the β subunit precipitates the holoenzyme and thus both subunits should be detectable (Fig. 2B). However, in contrast to Fig. 2A (immunoprecipitation with anti-CK2α antibody), Fig. 2B shows that the CK2 subunits were not detected equally well, despite the same antibody dilutions as used in Fig. 2A. We detected less CK2α than CK2β (Fig. 2B). This result is the same for serum-stimulated cells (S) and cisplatin-treated cells (Pt) (Fig. 2B).

The second interesting observation is that the amount of p53 precipitated by the anti-CK2β antibody is much higher than the amount pulled down by the anti-CK2α antibody (Fig. 2A) albeit the difference in the serum-stimulated cells is not that drastic with cisplatin treatment. About 3–5 times more p53 is precipitated from cisplatin-treated cell extracts than is the case from serum-stimulated extracts (Fig. 2B). The results, both with serum and with cisplatin treatment, support the notion of the existence of two CK2β populations, namely together with CK2α in a complex with p53 and CK2β by itself complexed to p53 (no CK2α present).

3.5. Immunoprecipitation with anti-L5 antiserum

Since p53, MDM2 and L5 were found to be immunoprecipitated in a complex we wanted to answer the question whether we could also find a complex involving L5, p53 and CK2. The experimental setup was as already described in the previous experiment. A polyclonal antibody directed against human protein L5 was applied. No significant difference was found when we looked for the presence of the CK2 subunits α and β (Fig. 2C) either in serum-stimulated or in cisplatin-treated cells. Immunodetection of p53 showed that p53 was present implying that the L5 antibody had precipitated L5, p53 and CK2 holoenzyme. There was significantly more p53 in the case of cisplatin-treated cells. So far we have no evidence for an in vivo association of MDM2 and CK2 using an anti-MDM2 antibody. Yet there is evidence for such a complex formation in the yeast two-hybrid system (Boldyreff et al., personal communication).

3.6. Characterization of high molecular mass complexes involving MDM2, p53, CK2

A cellular extract as was used for the immunoprecipitation experiments was analyzed on Superose 6 using the Pharmacia SMART system chromatographic equipment. The proteins of interest were detected by immunoblotting using specific antibodies. The first eight fractions did not contain any protein. Hence, Western blotting analysis was done from fractions 8–18. From the marker distribution the interesting fractionation range was between fraction 13 (thyroglobulin, 670 kDa) and fraction 19 (myoglobin, 17 kDa). Fig. 3A shows clearly that at 150 mM NaCl the p53 protein is distributed over a molecular mass range from 125 kDa to over 1000 kDa. CK2 was present mainly in fractions 14 and 15 which correspond to a molecular mass range of 300–500 kDa. But we also found a small amount of p53 protein in fraction 16 roughly at the position of the IgG, where the CK2 holoenzyme in an $\alpha_2\beta_2$ configuration would be expected. MDM2 is confined to fractions 16 and 17 corresponding to a molecular mass range of 125–200 kDa. The results described above were confirmed by immunoprecipitation studies using a polyclonal anti-CK2α and detecting L5 and p53 and by using an anti-CK2β antibody also detecting p53 and L5. The observed protein distribution profile shown in the immunoblots (Fig. 3A,B) corresponds to what we also saw after immunoprecipitation of the individual fractions supporting the notion that we are dealing with association and not merely with co-sedimentation (results not shown).

Fig. 3B shows the analysis in the presence of 1 M NaCl. Western blot was done from fractions 9–19. The MDM2 protein is shifted almost exclusively to fraction 17 which corresponds to M_r 150 000. But also in the case of the CK2 subunits we observed a distinct shift to the lower molecular mass species. CK2 is predominantly found in fractions 16 and 17, which is exactly the position where it would band as a hetero-tetramer. The same holds true for the β subunit. Only p53 was almost unchanged, i.e. sedimenting in a range from fractions 10 to 17 (vs. 9–17 at 150 mM NaCl). It is certainly true that a large amount of p53 was found to sediment in fraction 10 ($\gg$1000 kDa) at physiological salt concentration, whereas at 1 M salt the maximum seems to be at fractions 14 and 15 (400–600 kDa).

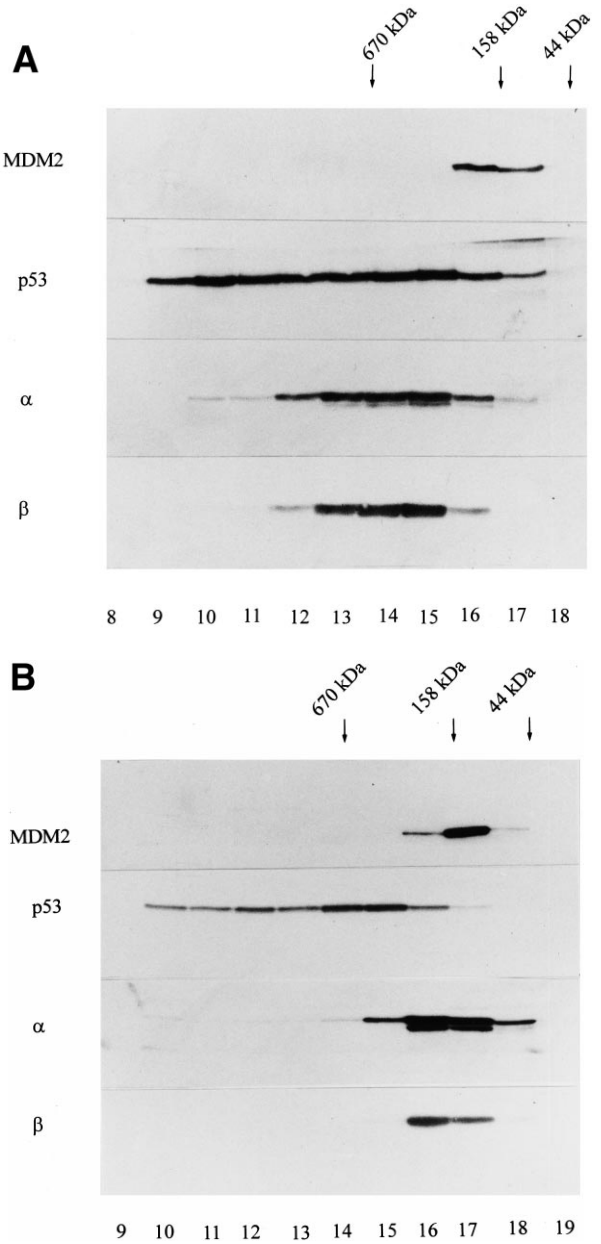

Fig. 3. A: Immunoblots from crude F9 extracts which had been cisplatin-treated for 4 h and then analyzed on Superose 6 gel filtration. The fractions were analyzed on SDS-PAGE and then transferred to membranes by Western blotting. The individual proteins were visualized by specific antibodies, e.g. anti-MDM2, anti-p53, anti-CK2α and β. The gel filtration was carried out in the presence of 0.15 M NaCl. B: Same as A, except that the gel filtration was carried out in the presence of 1 M NaCl.

4. Discussion

We wanted to study the influence of serum and cisplatin treatment on the expression and association of CK2 subunits α and β with proteins L5, MDM2 and p53. We applied immunodetection methods in order to follow the fate of p53 after serum stimulation and cisplatin treatment. In contrast to radioactive labeling of the cells, where predominantly protein new synthesis is measured, the immunological detection makes it possible to follow (i) the actual distribution and (ii) protein new synthesis. A prerequisite for the direct immunodetection of p53 in crude extracts by analysis on Western blots without prior labeling the cells, however, is the availability of a sensitive detection method. We have applied 1,2-dioxetan chemoluminescence substrates (Tropix, PE Applied Biosystems, 1997) which deliver a higher light intensity and hence a higher sensitivity in the detection of alkaline phosphatase (AP)-labeled immunocomplexes on blotting membranes [19]. Furthermore, an excess of substrate leads to light emission over 24 h and is directly proportional to the AP enzyme concentration. Hence, semi-quantitative determinations are possible.

The level of p53 in cells prior to serum stimulation and cisplatin treatment ($t = 0$ cells) was compared with the amount of p53 found after treatment. For a positive control we used extracts from SV80 cells where p53 is always found in a stable form owing to the presence of the large T antigen in these cells [20,21].

Before serum stimulation, cells were grown in the absence of serum for 2 days. Cisplatin treatment was initiated 2 days after seeding the cells and culturing in DMEM+10% FCS. There was no difference in the intensity of the MDM2 and CK2 subunit detection throughout the time course (Fig. 1A,B). However, p53 was affected. The activity of CK2 seems to be on average somewhat smaller in the cisplatin-treated cells than in the serum-stimulated cells. A paired *t*-test revealed a two-tailed *P* value of 0.0714, suggesting only marginal significance. Therefore, we believe that this activity difference is more apparent than real (Fig. 1A,B).

Thirty minutes after stimulation with serum one can see a drastic drop in the p53 level which is followed by an increase again. In serum-stimulated cells (Fig. 1A) the maximum level of p53 reached within 90 min is about four times higher than that seen in cells before serum treatment and it remains the same up to 240 min. In the case of cisplatin-treated cells (Fig. 1B) the p53 level increased steadily up to and beyond 240 min. After 240 min the amount of p53 was about eight times the value found for untreated cells. From the kinetics one can conclude that there are different mechanisms involved in the two processes, i.e. after serum stimulation and cisplatin treatment.

At first glance the results obtained here seem to differ from the data obtained from ^{35}S-labeled methionine cells and Western blot detection of p53 where a maximum level of p53 expression was found 18 h after serum stimulation [22]. We found a marked increase in the p53 level as early as 90 min after serum treatment with no further increase over the following 4 h. After this relatively short time Reich and Levine [22] had not detected any newly synthesized p53; only after 6 h a modest 10% of the maximum level was found. In the case of cisplatin treatment new protein synthesis via transcription can also be excluded because of the known effect of cisplatin as a DNA-damaging agent preventing any transcription. Therefore, it is very likely that the newly synthesized p53 protein comes from an internal mRNA pool. This notion is supported by data which showed in F9 cells that regulation of p53 expression occurs at the level of mRNA abundance and p53 protein stability [20]. In most of the cells investigated, also in 3T3 cells, the interaction of MDM2 with p53 leads to the proteolytic degradation of p53 with a relatively short half-life of p53, i.e. 0.4 h [20,23]. In F9 cells the half-life of p53 was determined to be 3 h [20–23] suggesting already an impairment of the usual proteolytic degradation process of p53. It is known that DNA damage leads to the activation of a DNA-dependent protein kinase which phosphorylates MDM2 and thus abolishes its p53 binding function [24], hence p53 is no longer funneled into the proteolytic degradation pathway and therefore increases steadily until the mRNA pool is exhausted.

In the case of the observed p53 increase following serum stimulation we observed a maximum level of p53 after 90 min. From then on no further p53 increase was observed. Obviously, another mechanism leads to the observed rapid increase in p53 protein. Since the MDM2 protein is not phosphorylated by DNA-dependent protein kinase owing to the lack of DNA damage we have to postulate that the newly synthesized p53 observed is not the result of protein new synthesis via transcription but rather the result of stored mRNA activation. It is known that the induction of certain proteins by serum is regulated at the level of translation [25–28].

In any case it is quite imaginable that the binding of p53 to other proteins, e.g. the large T antigen as in SVT2 cells, leads to an extended half-life of more than 22 h [20]. CK2 could provide a similar protective function of MDM2 phosphorylation by DNA-activated protein kinases after DNA damaging.

In order to answer the question whether CK2 subunits can be found associated with p53 in serum-stimulated and cisplatin-treated cells we carried out immunoprecipitation experiments.

4.1. IPα and β from cisplatin-treated cells

Let us now first consider the cisplatin-treated cells. As one can see in Fig. 2A the anti-α antibody precipitates both CK2 subunits in agreement with all evidence available so far that the majority of CK2 is present as the holoenzyme but also p53 is detected, unambiguous proof of an association of CK2 holoenzyme and p53.

In Fig. 2B we see the result of immunoprecipitation using an anti-CK2β antibody. In principle we expected the same result as seen in Fig. 2A. Yet, when we look at the result of the cisplatin-treated cells we see a change in the CK2 subunit ratio. Undoubtedly, more CK2β is now detected but also significantly more p53.

One might argue that the CK2 subunit signals were altogether weaker than in the case of IPα (Fig. 2A) suggesting that the antibody directed against the β subunit is poor compared to the α antibody. However, this is only more apparent than real because we deliberately reduced the exposure time because of the much stronger p53 signal observed in IPβ as compared to IPα seen in Fig. 2A. Therefore we believe that the anti-β antibody not only precipitates the CK2 holoenzyme/p53 complex but also a p53/β complex which would explain the observed differences in signal intensities.

4.2. IPα and β from serum-stimulated cells

In the case of the serum-stimulated cells we do not see a difference to the cisplatin-treated cells (Pt) in the case of IPα (Fig. 2A, lane S). Again when we look at IPβ (Fig. 2B) we see an imbalance in the subunit ratio as for the cisplatin-treated cells. However, there is much less p53 associated than is the case in the cisplatin-treated cells. This is also in agreement with the observations in the Western blots shown in Fig. 1A,B where the amount of p53 in cisplatin-treated cells 4 h after drug application is estimated to be four times higher. We postulate that beside the holoenzyme complex formation there should also be other complexes not involving the CK2 holoenzyme but also the CK2β and perhaps other so far unknown proteins.

The IPL5 from cisplatin-treated cells and after serum stimulation gave evidence for the association of CK2 holoenzyme (140 kDa), p53 (200 kDa) [29] and ribosomal protein L5 (34 kDa), a complex which would at least account for a total molecular mass of roughly 370 000 Da. However, as was the case with IPβ (Pt) the amount of p53 associated with CK2 in the cisplatin-treated cells was significantly higher suggesting the formation of CK2/p53 complexes by cisplatin treatment. In the light of the recent finding that CK2β can interact with Mos and A-Raf [30] the present finding does not come as a total surprise. What is, however, surprising is the association with ribosomal protein L5 that has previously been shown to be associated with MDM2/p53 complexes and MDM2 alone. Based on the observations by Guerra et al. [12] that CK2 phosphorylates MDM2 much better in the presence of p53 than in its absence we may speculate about the existence of higher molecular mass complexes in the cell involving CK2 (150 kDa), L5 (35 kDa), MDM2 (50–90 kDa), p53 (200 kDa). The complexes could reach sizes of 430–475 kDa. Indeed when we analyzed the extracts used for the IPs for microanalysis on a SMART system involving a Superose 6 gel filtration column the presence of p53 was detected in a molecular mass range from 150 000 to far over 1 million (the fractionation range for Superose 6 is from 5×10^3 to 5×10^6 Da). At physiological salt concentration CK2 was found in fractions over a molecular mass range from 200 kDa to over 1 million. MDM2 was found at 125–200 kDa allowing for a variety of different complex formations. However, one would not necessarily expect the *S*-value to be additive to the individual proteins making up the complex so that high molecular mass complexes would exhibit such *S*-values. Remarkable is the stability of the p53 complexes which are only marginally altered even at 1 M salt. As expected the complexes are mostly unstable in high salt. This is also clearly shown in Fig. 3B where only the p53 is still found at a molecular mass far above 1 million. CK2 and MDM2 have shifted to the lower sedimentation range.

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