Essay on total marks 45 assessment weighting 75

Parts of the World, European Union

Assignment 2 (Modules 4-6)

Background information:

The diagram below shows the activation of mTORC1 by growth factors as already encountered in workshop 5. The activity of mTORC1 is mainly controlled by the tuberous sclerosis complex, which consists of a heterodimer of tuberin (TSC2) and hamartin (TSC1). The known function of hamartin is to stabilise tuberin. Tuberin has GAP (GTPase activating activity) towards the G-protein Rheb. Rheb is active in its GTP-bound form and activates the mTOR kinase resulting in phosphorylation of S6Kinase (S6K) and consequently ribosomal protein S6, as well as 4EBP-1, resulting in protein synthesis and cell growth. Growth factor signalling results in activation of Akt which phosphorylates and inactivates TSC2, thus allowing Rheb and hence mTORC1 to be active.

The Tuberous sclerosis complex integrates multiple signals impinging on the cell and it is activated (thus deactivating Rheb and mTORC1) under conditions of stress. Thus the tuberous sclerosis gene products are tumour suppressors. Mutations in TSC2 (and TSC1) lead to multiple benign tumours (hamartomas) and abnormal growths in the brain resulting in epilepsy.
One major kinase which phosphorylates and activates TSC2 under stress conditions is AMP activated kinase (AMPK). AMPK in turn, is regulated in part by the kinase LKB1.
Another tumour suppressor, Ataxia-telangiectasia mutated (ATM) is a sensor of DNA damage (particularly double strand breaks caused by ionizing radiation and chemotherapeutic drugs). Deficiency of ATM leads to cancer

predisposition (particularly lymphomas) and neurodegeneration. ATM coordinates the cell cycle with damage-response checkpoints and DNA repair to maintain genomic integrity. It has also recently been implicated in metabolic regulation and response to reactive oxygen species. Cells deficient in either ATM or TSC2 exhibit defects in redox homeostasis.

ATM is a member of the phosphatidylinositol-3-kinase like kinase family (PIKK). Member s of this family, which includes mTOR, are large proteins (ATM is approx. 350kDa) and they are protein kinases rather than lipid kinases. In the inactive state, ATM has previously been shown to be dimer. On recruitment to DNA double strand breaks by the MRN complex, ATM dissociates into monomers and autophosporylates on Ser 1981. Ser 1981 phosphorylation has been used as a marker of ATM activation. The multitude of substrates of ATM include the tumour suppressors, p53 (Ser 15) and LKB1 (Thr 366) and the checkpoint kinase Chk2 (Thr68). Chk2 also phosphorylates p53 on Ser 15. A commonly used marker for ATM activation in the nucleus is Histone H2AX phosphorylation, and pH2AX can be visualised in foci at sites of DNA damage by using immunofluorescence techniques with anti-phospho H2AX antibodies.

Please refer to the following experimental data and answer the associated questions in your own words. In your answers make sure you refer to the panels in the figures to show your reasoning.

Answer in the spaces provided.

Figure 1: HEK293T cells were treated with 0. 5mM hydrogen peroxide, H2O2 for 30 min or irradiated with 5Gy of ionizing radiation (IR). Cell lysates were

analysed by SDS-PAGE and western blotting using phospho-specific antibodies as indicated.

Question 1: What conclusions can you draw from this set of experiments? (4 marks)

Question 2: What are the consequences of phosphorylation of Chk2 and p53? (4 marks)

In a different set of experiments MCF7 breast cancer cells were treated with peroxide at increasing concentrations and the cell lysates subjected to SDS-PAGE and western blotting with the antibodies shown. (ACC is a substrate of AMPK). Examine panel A in Figure2 below: -

Figure 2 panel A

Question 3: What further conclusions can be drawn about the effects of peroxide in these cells. (2 marks)

Panels B, C and D show similar experiments performed in whole cell lysates as well as cytoplasmic and nuclear extracts and cells treated with leptomycin B.

Figure 2 continued

Question 4: What further information do these sets of experiments provide? (4 marks)

Question 5: What is the purpose of using leptomycin B? (1 mark)

Question 6: Why were antibodies to lamin and LDH (lactate dehydrogenase) used in these blots? (2 marks)

HeLa cells lack LKB1. LKB1 was therefore overexpressed in these cells in the wild-type form and with a T366A mutation (the site phosphorylated by ATM).

Figure 3. In panel A HEK293T cells were immune precipitated with antibodies to LKB1 and western blotting was carried out with antibodies to phosphoLKB1(Thr 366). (NT = untreated, IR = irradiated, IP = immunoprecipitated)

In panel B: HeLa cells and HeLa cells overexpressing LKB1 (wt and mutant) were treated with peroxide.

Question 7: What conclusion can you draw from these experiments? (4 marks)

AMPK phosphorylates TSC2 at two sites T1271 and S1387 to activate it. Cells deficient in TSC2 were transfected with wild type TSC2 and TSC2 with these 2 sites mutated to alanine (TSC2AMPK2A).

Figure 4: Panel A shows the effect of peroxide on wild type and TSC2 deficient cells. Panel B shows the effect in TSC2 deficient cells transfected with wild type or AMPK2A mutant TSC2.

Question 8: From the information gained from all of these experiments draw a diagram of the signalling pathway from ATM activation after treatment with peroxide and indicate in which cellular compartment this pathway is occurring. (5 marks)

Nuclear envelope

Suppression of mTOR activity leads to autophagy, a means for cell survival under stress and also an alternative cell death pathway.

Atm deficient mice are very susceptible to tumours, particularly lymphomas. The onset of tumours can be delayed and survival or mice can be prolonged by feeding the mice antioxidants (CTMIO) or rapamycin as shown below.

Red (atm+/-) heterozygote

Blue atm(-/-) plus rapamycin

Green atm (-/-) no treatment

Question 9: Based on your conclusions from all the previous questions, provide a molecular explanation for the effectiveness of antioxidants and rapamycin in delaying tumour onset in atm deficient mice. (4 marks)

A major question arising from these studies is how ATM is activated after oxidative stress. The following questions deal with this aspect.
As shown above in Figure 1, when HEK293T cells were treated with hydrogen peroxide, ATM autophosphorylation was activated as was its kinase activity for other substrates including p53, Chk 2 and other targets (see Fig 1 and other relevant results above)
These and other data presented above suggest that ATM may be subject to some form of oxidative modification that influences its kinase activity. To further investigate this, and in an attempt to identify the nature of any oxidative modification, the ATM protein was purified and the following experiments were performed providing the results shown below.

Figure 6: Kinase activity of purified ATM assayed using p53 as the substrate was tested in the absence (-) and presence of increased concentrations of hydrogen peroxide 0. 0625, 0. 125, 0. 25, 0. 5 and 1mM. Phosphorylation of

the p53 was detected by SDS PAGE and Western Blot using an antibody to p53-phospho-Ser-15.

Question 10. What conclusion(s) do you draw from the results above? (2 marks)

Figure 7

Kinase assays were performed as in Fig 6 above. The first sample of ATM was assayed in the absence of hydrogen peroxide while the second sample was assayed in the presence of hydrogen peroxide (0. 81mm). The third and fourth samples of ATM were treated with 0. 25 and 0. 5mM NAC (N-acetyl cysleine) for 15 minutes before being assayed in the presence of 0. 81mM hydrogen peroxide.

Question 11. What conclusion(s) do you draw from the results above? (2 marks)

Q 10. What conclusion(s) do you draw from the results above?

Figure 8

Purified ATM was treated with 0, 0. 125mM, 0. 25mM or 0. 5mM hydrogen peroxide then analysed by SDS PAGE in the presence (+) and absence (-) of β-mercaptoethanol (BME) and analysed by western blotting using an antibody to ATM. M approx 350K, D approx 700K in MW

Question 12. What conclusion(s) do you draw from the results above? (2 marks)

As a result of considering the data above the investigators then made a range of mutant ATM’s in which different individual cysteine residues were

replaced by leucine (L) residues. Of all these single cys-leu mutant proteins all but one demonstrated activation of the kinase activity on treatment with hydrogen peroxide. The only one that failed to be activated by hydrogen peroxide was the C2991L mutant of ATM.

Question 13. What conclusions do you draw from these results? (2 marks)

Question 14. Based on the data provided above, describe the most likely oxidative modification to ATM that is induced by hydrogen peroxide treatment. (4 marks)

Question 15. The oxidation clearly activated the kinase activity of ATM with the subsequent activation of the downstream pathways. To act as a redox control switch for control of ATM dependent signalling pathways in the cell, the cell would need to have a mechanism to reverse this oxidative modification.

Describe the most likely cellular enzyme system that could catalyse the reversal of this oxidative modification to ATM. (3 marks)