The UIM-containing protein RAP80/UIMC1 functions in DNA damage repair response. Jun Yan, Yong-Sik Kim, Xiao-Ping Yang, Li-Ping Li, Grace Liao, Fen Xia, and Anton M. Jetten

Supplement

Materials and Methods

Plasmids. pLXIN and pEGFP were purchased from BD Biosciences. pLXIN-3×FLAG-RAP80, pLXIN-3×FLAG-RAP80ΔUIM, pLXIN-3×FLAG-RAP80 mutants (aa 1-582, 1-504, 1-404, 1-204, and 1-122) and pEGFP-RAP80 were described previously (1, 2). The plasmid pEGFP-RAP80ΔUIM was generated by PCR amplification. The regions up- and down-stream from the UIMs were first amplified by PCR, then ligated at the introduced *Xho*I sites, and subsequently inserted into the *Eco*RI and *Bam*HI sites of pEGFP. The pEGFP-RAP80 deletion mutants, encoding the regions between aa 1-404, 1-304, 1-204, 1-122, and 1-78, were generated by inserting the corresponding PCR products into the *Eco*RI and *Bam*HI sites of pEGFP-C3. pGEX-RAP80(aa168-405) and pGEX-RAP80(413-500) were constructed by inserting the corresponding region of RAP80 into the *Bam*HI and *Eco*RI sites of pGEX-5x-3. The pEGFP-RAP80 mutants A88S, A113S, A88S/A113S, S205G, S402A, and S205G/S402A and the pGEX-RAP80(168-405) mutants S171G, S205G, T228A, S402A, and S205G/S402A were generated using a Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pEGFP-RAP80 (8MT) with all potential ATM phosphorylation sites (S101, S140, S171, S205, T228, S402, S419, and T448) mutated was generated with a Quickchange multi site-directed mutagenesis kit (Stratagene). The sequence of each insert was verified by DNA sequencing. The pcDNA3-FLAG-ATM expression vectors encoding wild type

(WT) or kinase dead (KD) ATM were gifts from Dr. Michael Kastan (St. Jude Children's Research Hospital, Memphis, TN). The pcDNA3-Myc-BRCA1 and pcDNA3.1-HA-BRCT encoding the BRCT domain of BRCA1 were kindly provided by Dr. Jane E. Visvader (Walter and Eliza Hall Institute of Medical Research and Bone Marrow Research Laboratories, Melbourne, Australia) (3).

ATM kinase assay. *E. coli* BL21 cells (Stratagene) transformed with pGEX-5-3 plasmid DNA encoding wild type or mutant GST-RAP80(168-405), were grown at 37°C to mid-log phase. Synthesis of GST-RAP80 fusion proteins was then induced by the addition of isopropylthiogalactopyranoside (IPTG; 0.5 mM final concentration) at 37°C. After 4 h of incubation, cells were collected, resuspended in BugBuster protein extraction reagent (Novagen, Madison, WI) and processed according to the manufacturer's protocol. Cellular extracts were then centrifuged at 15,000 x g, and the supernatants containing the soluble GST-RAP80 fusion proteins collected. Phosphorylation of GST-RAP80 proteins by ATM kinase was determined following a procedure described by Ziv et al. (4). Briefly, HEK293T cells transfected with pcDNA3-FLAG-ATM or pcDNA3-FLAG-ATM(KD) encoding a kinase dead (KD) ATM mutant, were lysed in DM lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% dodecyl maltoside, 5 mM EDTA, 50 mM NaF, and protease and phosphatase inhibitor cocktails (Sigma). FLAG-ATM proteins were isolated by incubating lysates for 2 h at 4°C with anti-FLAG M2 affinity resin. Immune complexes were collected and washed three times with lysis buffer and twice with kinase buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 4 mM $MnCl_2$, 10% glycerol, 1 mM dithiothreitol, plus protease and phosphatase inhibitor cocktails). Reactions were carried out in kinase buffer containing 20 μM ATP, 10 μCi of [γ-^{32}P]ATP (GE

Healthcare Bio-science, Piscataway, NJ), and 1 μg GST-RAP80 substrate for 15 min at 30°C. The reaction was stopped with SDS sample buffer, samples boiled for 10 min, and proteins separated by electrophoresis on a 4-12% gradient SDS-PAGE gel. Phosphorylated proteins were detected by autoradiography.

References

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2. Yan J, Kim S-K, Yang X-P, Albers M, Koegl M, Jetten AM. Ubiquitin interacting motifs of RAP80 are critical in its regulation of Estrogen Receptor α. Nucl Acids Res 2007.
3. Sum EY, Peng B, Yu X, *et al.* The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. j Biol Chem 2002;277:7849-56.
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Legends

Figure 1. MCF-7-RAP80 cells, which stably express 3×FLAG-tagged RAP80, were exposed to γ-irradiation and 3 h later stained with anti-3×FLAG, anti-γ-H2AX antibody, and DAPI. The subcellular distribution of 3×FLAG-RAP80 and γ-H2AX was examined by confocal microscopy.

Figure 2. Phosphorylation of RAP80 by ATM is not required for its translocation to IRIF. *A.* MCF-7 cells were transfected with pEGFP-RAP80 plasmid DNA encoding wild type human RAP80 or RAP80 in which all 8 potential ATM-phosphorylation sites were mutated. After 48 h incubation cells were treated with γ-irradiation (10 Gy) and 3 h later stained with anti-γ-H2AX antibody. Localization of EGFP-RAP80 and γ-H2AX was examined by confocal microscopy. *B.* A-T cells (GM05823) and control cells (GM05757) were infected with retrovirus encoding FLAG-RAP80. After 48 h infection cells were treated with γ-irradiation (10 Gy) and 3 h later stained with anti-FLAG and anti-γ-H2AX antibodies. Localization of FLAG-RAP80 and γ-H2AX was examined by confocal microscopy.