Analysis of DNA ligase IV mutations found in LIG4 syndrome patients: the impact of two linked polymorphisms

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LIG4 syndrome patients have hypomorphic mutations in DNA ligase IV. Although four of the five identified patients display immunodeficiency and developmental delay, one patient was developmentally normal. The developmentally normal patient had the same homozygous mutation (R278H) in DNA ligase IV as one of the more severely affected patients, who additionally had two linked polymorphisms. Here, we examine the impact of the mutations and polymorphisms identified in the LIG4 syndrome patients. Examination of recombinant mutant proteins shows that the severity of the clinical features correlates with the level of residual ligase activity. The polymorphisms decrease the activity of DNA ligase IV by ~2-fold. When combined with the otherwise mild R278H mutation, the activity is reduced to a level similar to other LIG4 patients who display immunodeficiency and developmental delay. This demonstrates how coupling of a mutation and polymorphism can have a marked impact on protein function and provides an example where a polymorphism may have influenced clinical outcome. Analysis of additional mutational changes in LIG4 syndrome (R580X, R814X and G469E) have led to the identification of a nuclear localization signal in DNA ligase IV and sites impacting upon DNA ligase IV adenylation.

INTRODUCTION

Genomic stability is important for cell growth and cancer avoidance. A plethora of damage response mechanisms collectively act to maintain genomic stability in the face of continuous DNA damage incurred by exogenously and endogenously generated DNA damaging agents. The importance of these mechanisms is underscored by the fact that several hereditary syndromes associated with developmental abnormalities and cancer predisposition harbour mutations in essential components of these pathways. Although the hereditary syndromes are conferred by mutations that have profound impact on protein function, it has been suggested that less impacting mutations, including polymorphisms, may contribute to instances of sporadic cancer. Considerable resources have been invested in epidemiological studies searching for polymorphisms that correlate with cancer predisposition. Although few candidates have been identified, it has been suggested that combinations of polymorphisms may also be important.

LIG4 syndrome (OMIM 606593) is an autosomal hereditary disorder conferred by mutations in DNA ligase IV, a protein that plays a pivotal role in DNA non-homologous end-joining (NHEJ) and V(D)J recombination (1). NHEJ is the major mechanism in mammalian cells for DNA double strand break (DSB) repair (2,3). DSBs are also introduced endogenously during the process of V(D)J recombination (4). Six proteins function in NHEJ and V(D)J recombination, namely, Ku70, Ku80, DNA-PKcs, DNA ligase IV, XRCC4 and Artemis. DNA ligase IV co-associates with XRCC4 and functions in the final joining step of NHEJ (5–7). DNA ligase IV has a conserved ligase domain at its N-terminal and two C-terminal BRCT domains. Interaction with XRCC4 occurs via the region that lies between the two BRCT domains (8,9). The first step of the ligation reaction involves formation of a ligase–adenylate complex, which requires a highly conserved motif, KYLGER, located in the N-terminal region of the protein. DNA ligase IV exists as a pre-adenylate complex in mammalian cells (10). The second
step of the reaction involves transfer of the AMP moiety to the DNA end. Finally, ligation occurs with release of non-adenylated ligase.

As DNA ligase IV is essential for development in mice, mutations in the LIG4 syndrome patients are likely to be hypomorphic (11,12). Five LIG4 syndrome patients have been described (13,14). One individual, identified by his over-response to radiotherapy, was clinically normal until the onset of leukaemia at age 14, whereas the remaining patients displayed pancytopenia, slow development and growth delay. Strikingly, the clinically normal patient carried the same homozygous mutation (R278H) in DNA ligase IV as one of the more severely affected patients, whose DNA ligase IV sequence differed solely by the additional presence of two linked polymorphisms (found at frequencies of 7 and 19%) (15). Previously, we have shown that the R278H mutation impacts upon DNA ligase IV function reducing the activity to ~10% of wild-type (WT) levels. Here, we examine the impact of the additional mutations and the polymorphisms identified in the LIG4 syndrome patients using sensitive and quantitative assays for distinct aspects of DNA ligase IV function. Our findings demonstrate that the two linked polymorphisms in DNA ligase IV impact upon function. Although the effect is not dramatic in a WT DNA ligase IV protein, the impact becomes significant when coupled with the R278H mutational change and likely underlies the difference in clinical severity between the LIG4 syndrome patients. This provides an example of a polymorphic change affecting protein function with a likely link to clinical outcome. Our findings also provide insight into functionally important sites in DNA ligase IV.

RESULTS

Two closely linked N-terminal LIG4 polymorphisms mildly impair adenylation and ligation activity, but have a large impact when coupled with the R278H mutation

Figure 1 shows the sites of mutational changes identified in the LIG4 syndrome patients (13). Patients 180BR and 411BR have the same R278H homozygous mutation which reduces DNA ligase IV adenylation and ds ligation activities to ~10% of the WT activity (16). Patient 411BR has, in addition, two closely linked N-terminal polymorphic changes resulting in two amino acid substitutions. To examine the impact of these polymorphisms, we generated cDNAs expressing LIG4 protein harbouring the two N-terminal polymorphisms (A3V + T9I LIG4), the R278H mutation (R278H LIG4) and the three changes together (A3V + T9I + R278H LIG4); co-expressed them with XRCC4 cDNA in baculovirus and used them to quantitatively monitor DNA ligase IV activity. Neither the R278H mutation nor the combined A3V and T9I changes affect interaction with XRCC4 (14). Consistent with previous results, we observed low but residual adenylation activity (~5% of WT activity) with the R278H LIG4 protein (16) (Fig. 2A). The A3V + T9I protein showed reduced adenylation activity compared with WT LIG4 protein (~50% of the WT activity) (Fig. 2A).

In contrast, no residual adenylation activity could be detected with the A3V + T9I + R278H LIG4 protein (Fig. 2A).

We next examined ds ligation activity. Similar to the findings for adenylation activity, decreased ds ligation activity was observed with the A3V + T9I LIG4 protein (Fig. 2A). Since the assay ceases to be linear >500 ng WT protein, the analysis was repeated using lower protein concentrations and 2–3-fold decreased activity was observed (Fig. 2C and D). Consistent with previous findings, the ds ligation activity of the R278H mutant protein was 5–10% of WT activity (Fig. 2A and D) (16). In contrast, no residual ds ligation activity was detectable using the A3V + T9I + R278H LIG4 protein even up to 1000 ng protein (Fig. 2A and D). The viability of the 411BR patient indicated that the A3V + T9I + R278H LIG4 protein should retain some residual activity. We, therefore, examined the triple mutant protein using a nick ligation assay, which has greater sensitivity than the ds ligation assay and low residual activity was detected (Fig. 2B). The activity using 1000 ng mutant protein was similar to that obtained with 10 ng WT protein suggesting that the mutant protein has ~1% of the WT LIG4/XRCC4 activity. These findings show that the two N-terminal polymorphisms slightly impair the ligation and adenylation activities of DNA ligase IV. Moreover, when combined with the R278H mutation, the decrease in protein function is at least additive.

Analysis of additional mutant proteins identified in the LIG4 syndrome patients: identification of a nuclear localization signal in DNA ligase IV

The findings mentioned above raise the possibility that the more severe clinical features of patient 411BR relative to 180BR might be attributable to a lower level of residual activity. Two further LIG4 syndrome patients displayed marked developmental abnormalities. We, therefore, examined the mutational changes identified in these patients.

One factor that might influence DNA ligase IV function is its ability to enter the nucleus. We, therefore, examined localization of the mutant proteins. cDNAs encoding enhanced green fluorescent protein (EGFP) or a myc epitope were fused in frame to the amino-terminal of WT or mutant LIG4...
cDNAs expressing the mutations R580X, R814X and G469E. Since XRCC4 has its own nuclear localization signal (NLS), we examined localization of DNA ligase IV in the absence of XRCC4 following transient transfection of the plasmids in XR-1 (XRCC4<sup>−/−</sup>) cells (17). Localization was examined by direct fluorescence of EGFP-tagged protein. Western blotting showed that immunoreactive proteins with the anticipated molecular weight were expressed (data not shown). EGFP alone was located in the nucleus and cytoplasm consistent with previous findings that EGFP can diffuse into the nucleus. EGFP–WT, G469E and R814X LIG4 proteins localized predominantly to the nucleus, whereas EGFP–R580X LIG4 localized exclusively to the cytoplasm (Fig. 3A). Similar results were obtained using myc-tagged LIG4 proteins (data not shown). These findings demonstrate that the C-terminal region of LIG4 (residues 580–911) either encompasses an NLS or interacts with another protein which targets it to the nucleus. To define the region required for localization, we examined D<sub>653–911</sub> LIG4, which encompasses additional residues C-terminal to the R580X LIG4 truncation. LIG4 nuclear localization was now observed (Fig. 3). Two sequential potential NLSs were identified in LIG4 designated NLS1 (P<sub>623</sub>–I<sub>629</sub>) and NLS2 (A<sub>630</sub>–P<sub>638</sub>). cDNAs encoding NLS1, NLS2 and the tandem NLS (P<sub>623</sub>–I<sub>638</sub>) were cloned into the EGFP-tagged vector and transiently transfected into XR-1 cells. NLS1 and NLS2 localized predominantly to the nucleus although fluorescence was also observed in the cytoplasm (Fig. 3A). In contrast, the tandem NLS showed marked and exclusive nuclear localization. To verify the function of either encompasses an NLS or interacts with another protein which targets it to the nucleus. To define the region required for localization, we examined Δ<sub>653–911</sub> LIG4, which encompasses additional residues C-terminal to the R580X LIG4 truncation. LIG4 nuclear localization was now observed (Fig. 3). Two sequential potential NLSs were identified in LIG4 designated NLS1 (P<sub>623</sub>–I<sub>629</sub>) and NLS2 (A<sub>630</sub>–P<sub>638</sub>). cDNAs encoding NLS1, NLS2 and the tandem NLS (P<sub>623</sub>–I<sub>638</sub>) were cloned into the EGFP-tagged vector and transiently transfected into XR-1 cells. NLS1 and NLS2 localized predominantly to the nucleus although fluorescence was also observed in the cytoplasm (Fig. 3A). In contrast, the tandem NLS showed marked and exclusive nuclear localization. To verify the function of
the tandem NLS, a mutated LIG4 cDNA (EGFP–MutNLS LIG4) was constructed to encode a LIG4 protein in which the lysines (K) 626, 627, 629, 633, 635 and 636 (PQEK626K627RK629AAPK633MK635K636VI) were mutated to threonine residues. Following transfection into XR-1 cells, EGFP–MutNLS LIG4 failed to localize to the nucleus (Fig. 3A). We also examined localization of the LIG4 MutNLS protein in the presence of XRCC4. EGFP–MutNLS, which failed to enter the nucleus in the absence of XRCC4, entered the nucleus when co-transfected with myc–XRCC4 (Fig. 3B). We also generated an XRCC4 construct that lacked a previously described NLS, designated delNLS XRCC4 (17). Neither EGFP–delNLS XRCC4 nor myc–delNLS XRCC4 localized to the nucleus in contrast to EGFP–WT XRCC4 (17) (Fig. 3B and data not shown). Co-transfection of EGFP–delNLS XRCC4 with myc–WT LIG4 demonstrated that XRCC4 lacking its own NLS was targeted to the nucleus when complexed with WT LIG4 (Fig. 3B). Together, these results show that a bipartite NLS at position 623–638 can target DNA ligase IV to the nucleus. This NLS has a redundant role with an NLS present in XRCC4. The R580X LIG4 protein lacks both this region and the XRCC4 interaction region and thus fails to localize to the nucleus even in CHO-K1 cells that express XRCC4 (Fig. 3C). We, therefore, consider that R580X represents a null mutation and it was not subject to further analysis.

Analysis of R814X LIG4: loss of the second BRCT domain impairs interaction with XRCC4, adenylation and ligation activities

The region of LIG4 located between the two BRCT domains (residues 751–800) has been reported to be necessary and sufficient for XRCC4 binding (8). Although the R814X truncation leaves this region intact, we had previously observed decreased R814X LIG4/XRCC4 interaction using proteins expressed by rabbit reticulocyte lysates (14). To examine interaction in vivo, we stably expressed either myc-tagged WT or R814X LIG4 in XR-1 cells, followed by transient transfection with either EGF or EGFP-tagged XRCC4. WT and R814X LIG4 were expressed to similar extents in cells expressing or not expressing XRCC4 as shown by direct western blotting with anti-myc antibodies (Fig. 4A; WB: α-myc, lanes 3, 4, 7, 8, 11 and 12). Immunoprecipitates obtained using anti-myc antibodies were examined using anti-myc (DNA ligase IV) and anti-EGFP (XRCC4) antibody. Although equal levels of DNA ligase IV were present in all the immunoprecipitates (Fig. 4A, lanes 1, 2, 5, 6, 9 and 10), the level of XRCC4 co-immunoprecipitated by R814X LIG4 was <10% of the level obtained with the WT LIG4 protein (Fig. 4A, compare lanes 1, 5 and 9). XRCC4 was efficiently expressed in the R814X cells (Fig. 4A, lanes 3, 7 and 11). These results demonstrate that partial loss of the second BRCT domain significantly impairs interaction with XRCC4 in vivo.

We next examined hamster-expressed R814X LIG4 for adenylation activity. Co-expression of XRCC4 stimulated adenylate complex formation by myc–WT LIG4 (Fig. 4B, lanes 1 and 2) confirming previous findings that XRCC4 stimulates but is not essential for LIG4 adenylation activity (5,19). R814X LIG4 was dramatically reduced for adenylation activity both in the presence or in the absence of XRCC4 expression (Fig. 4B, lanes 3 and 4). The markedly reduced adenylation activity in the absence of XRCC4 demonstrates that an intact tandem BRCT domain stimulates ligase IV–adenylate complex formation separately from any impact of XRCC4. Whether the reduced adenylation in the presence of XRCC4 is simply due to reduced interaction or a combination of reduced interaction and adenylation.
capacities cannot be determined. Thus, the extreme C-terminal of DNA ligase IV is required for efficient interaction with XRCC4 as well as efficient adenylate complex formation.

We also examined baculovirus expressed R814X LIG4. Our previous studies have shown that co-expression of LIG4 and XRCC4 is required for LIG4 solubility (16). WT LIG4 or R814X LIG4 cDNAs were co-expressed with XRCC4 cDNA. The size of both the WT and mutant-expressed complexes were estimated by size fractionation through a range of spin columns and all of the LIG4 protein was recovered in the size range anticipated for LIG4/XRCC4 complexes (data not shown). Additionally, Coomassie blue staining of an SDS-PAGE gel showed that XRCC4 and DNA ligase IV were present at similar ratios in the expressed WT and mutant protein (shown in Fig. 5A for R814X LIG4 protein). Thus, in contrast to the hamster cell expression system, which permits the analysis of DNA ligase IV activity in the absence of XRCC4, only LIG4 complexed with XRCC4 is analysed in the baculovirus expression system. Examination of the R814X LIG4/XRCC4 complex showed no detectable residual adenylate activity in contrast to the reduced but residual activity detectable in hamster cells (Fig. 5B).

Since ds ligation is carried out in the absence of ATP, only pre-adenylated LIG4/XRCC4 complexes are monitored. Surprisingly, the R814X LIG4/XRCC4 complex, despite having barely detectable adenylate activity, has ds ligation activity that is ~10–15% of the WT activity (Fig. 5C). This result suggests that the R814X mutation has a bigger impact upon adenylate activity of LIG4 than it has upon its ligation activity (see Discussion).

**G469E LIG4 dramatically affects DNA ligase IV–adenylate complex formation**

Expression of EGFP–XRCC4 in XR-1 cells stably expressing myc-tagged G469E LIG4 showed that G469E LIG4 interacts normally with XRCC4 (Fig. 4A, lanes 9 and 10). However, analysis of the anti-myc immunoprecipitates for DNA ligase IV–adenylate complex formation demonstrated a dramatic impact with no residual activity detectable either in the absence or in the presence of XRCC4 (Fig. 4B, lanes 5 and 6). Analysis of the G469E LIG4/XRCC4 complex expressed in baculovirus for adenylate complex formation and ds ligation activity also demonstrated a dramatic impact with no detectable residual activity (Fig. 5B). Examination of the G469E LIG4 using the nick ligation assay demonstrated low but residual activity, which was close to the limit of detection (Fig. 5C). Together, these results show that the G469E mutation does not impair interaction with XRCC4 but dramatically impacts adenylate and ligation activity.

**DISCUSSION**

**Clinical severity correlates with the level of residual DNA ligase IV activity**

Patient 180BR developed leukaemia but was otherwise clinically normal, although the remaining LIG4 syndrome patients display pancytopenia, developmental and growth delay (2303, 2304, 99P0149 and 411BR). R278H, the mutant protein expressed in 180BR, had 5–10% residual adenylate and ligation activities as observed previously (16). In contrast, the more severe immunodeficient patients carried more severely impacting mutational changes (Table 1). The R580X mutation is likely to represent a null allele since the protein is not stably expressed, does not interact with XRCC4 and does not enter the nucleus. The R814X mutant protein retained ~10–15% residual ds ligation activity. However, DNA ligase IV protein was barely detectable in patient 2303 indicating that in vivo the R814X truncated protein shows reduced expression most likely due to non sense mediated decay (14). Thus, we estimate that the combined impact of decreased expression and activity of the R814X mutant protein results in <1% residual activity in vivo. The G469E mutation present in patient 99P0149 and combined mutations present in patient 411BR
(A3V + T9I + R278H) yield proteins with no detectable residual adenylation or ds ligation activity. Less than 1% residual activity is detectable by the more sensitive nick ligation assay. Taken together, these findings provide strong evidence that the clinical severity of the LIG4 syndrome patients correlates with the severity of the mutational changes they harbour. This suggests that the R278H is a milder mutation allowing clinically normal development although potentially conferring a predisposition to leukaemia.

Two linked polymorphisms affect DNA ligase IV function

Two linked polymorphisms in the N-terminal of DNA ligase IV mildly but reproducibly reduce adenylation and ligation activities (~2–3-fold). Importantly, the impact is at least additive when coupled with the R278H mutation. Thus, the R278H mutant protein allows 5–10% residual adenylation activity, yet no residual activity is detectable in the R278H + A3V + T9I mutant protein. We estimate that our assays are capable of detecting 1–2% WT activity. A similar result is observed for ds ligation activity. One explanation for the effect of the polymorphisms is that they impair stacking interactions between residues that help to maintain protein confirmation, which may be additive to the impact of mutations elsewhere in the gene.

These findings correlate with the severity of the patients’ clinical features. We propose that although the residual activity present in 180BR is sufficient for normal development, the level present in the remaining patients falls below the threshold required for normal development. Our findings strongly suggest, therefore, that the presence of the polymorphisms underlies the difference in clinical features between patients 180BR and 411BR and, thus, have conferred a marked clinical impact. The A3V and T9I polymorphisms have been described previously and have allele frequencies of 0.07 and 0.19, respectively (dbSNPcluster id rs1806389 and rs1805388, respectively) (15). We do not have cell lines from individuals homozygous solely for both polymorphisms. However, a small number of such individuals were identified, which lie within one of two domains, designated 1 and 2 (reviewed in 20), which encompass the ATP-binding

Important domains in DNA ligase IV

First, analysis of the R580X mutation has led to the identification of a novel NLS in DNA ligase IV that functions in concert with an NLS in XRCC4 to ensure nuclear localization of the DNA ligase IV/XRCC4 complex (17).

Second, analysis of the R814X mutation has shown that the BRCT domains impact upon DNA ligase IV adenylation. BRCT domains are present in NAD+-dependent ligases, as well as mammalian DNA ligases III and IV (20). The R814X mutation results in loss of the second BRCT domain leaving the linker region, which is required for XRCC4 interaction, intact (8,9). However, R814X LIG4 shows impaired interaction with XRCC4 observable in vitro and in vivo (Fig. 4) (14). In contrast, Grawunder et al. (8) reported previously that an R800X LIG4 protein interacted normally with XRCC4. Our results suggest that the presence of a partial second BRCT domain can inhibit interaction raising the possibility that a function of the two BRCT domains might be to bring the interacting region into a structural context that allows XRCC4 binding.

R814X LIG4 is significantly impaired in adenylation complex formation. Our findings confirm previous studies that interaction between DNA ligase IV and XRCC4 enhances but is not essential for ligase IV adenylation (5). However, impaired adenylation complex formation is observed with the R814X LIG4 protein even in the absence of XRCC4, demonstrating that an intact BRCT tandem motif is required for efficient adenylation in a manner distinct from its requirement for XRCC4 interaction. Our results do not allow us to distinguish whether pre-adenylated R814X protein has decreased ds ligation activity or whether the entire impact on ligation can be attributed to impaired ability to form an adenylation complex.

Finally, analysis of G469E provides a further demonstration that sites distant from the adenylation domain can dramatically affect DNA ligase IV adenylation activity. Six conserved motifs (designated I–VI) within DNA ligases have been identified, which lie within one of two domains, designated 1 and 2 (reviewed in 20), which encompass the ATP-binding
site and residues essential for ligation, respectively. The G469E mutational change lies outside of these conserved motifs (I–VI) within domain 2. However, a sequence alignment of the region encompassing G469E shows that this region is highly conserved between eukaryotic DNA ligases and has a consensus motif, GAYYGKGK. The dramatic impact of the G469E mutation on adenylation raises the possibility that this is an important catalytic motif. A mutation identified in DNA ligase I in another immunodeficient patient, which lies close to the equivalent residue of G469, did not affect adenylate complex formation, but it did affect the formation of a nicked DNA–AMP complex, the next stage of the ligation reaction (21). Further work is in progress to examine the impact of the C-terminal region and this conserved motif in the OB domain on DNA ligase IV function.

Considerable effort has been invested in examining the impact of polymorphisms on protein function, on cancer predisposition and on disease. Although few polymorphisms that impact upon protein function have been identified, the studies are frequently limited by the lack of sensitive assays. Here, we identify two linked polymorphisms that mildly impact upon function. More importantly, we show that when coupled with a second mildly impacting mutational change, there is an additive impact that likely reduces the protein activity to a level below that required for normal development. We present evidence suggesting that the presence of the polymorphisms has profoundly affected the development of an individual. Our study represents a striking example of the compounding effects of distinct mutational changes in a protein and provides a framework for considering the impact of multiple polymorphisms, where the level of residual activity may be greater and the impact may lie not in cancer development, but on cancer predisposition.

MATERIALS AND METHODS

Cloning of human DNA ligase IV (LIG4) and XRCC4 cDNAs into mammalian expression vectors

myc cDNA encoding a single myc epitope was introduced into pCI-neo vector (Promega) at the Nhel and Xhol restriction sites generating a pCI-neo(myc) vector. The HpaI–BamHI fragment, which contains the neomycin resistance gene, was replaced by a PvuII–BamHI fragment, containing the puromycin gene, from a pPUR vector (CLONTECH laboratories, Inc., CA, USA) generating a pCI-puro(myc) vector. The mutations (1738C/T, 2440C/T and 1406G/A) were introduced into cDNA (accession number NM002312) by site-directed mutagenesis (14). Wild-type and mutated cDNAs were then either sub-cloned into the pCI-puro(myc) vector generating pCI-puro(myc)WT, -R580X, -R814X or -G469E LIG4 or into the pEGFP-C3 vector generating the same constructs with an EGFP tag. These pCI-puro(myc) vectors were stably transfected into XR-1 cells. pCI-puro(myc)WT-LIG4 was digested with HpaI and Xhol and the ~2 kb fragment was inserted into the pEGFP-C3 vector following digestion by SmaI and Xhol, leading to pEGFP–LIG4Δ653–911. The construct mutated at the nuclear localization site was generated by PCR using specific primers. The human full length XRCC4 cDNA or the PCR-generated construct lacking the last 69 amino acids were cloned into pCI-puro(myc) or pEGFP-C3 generating pCI-puro(myc)hXRCC4, pEGFP–hXRCC4, pCI-puro(myc)hXRCC4ΔNLS and pEGFPPhXRCC4ΔNLS vectors, respectively.

Cell culture and transfection

CHO-K1 (WT) and XR-1 (XRCC4 deficient) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, streptomycin 5 U/ml, penicillin 100 U/ml, glutamine 2 mM and non-essential amino acid (Gibco-BRL, Life Technology). Cells were transfected when 50–70% confluent using Genejuicetm (Novagen). Cells were recovered 48 h later or selected for stable clones by addition of puromycin (Sigma) at 5 μg/ml for 10 days. Clones were analysed for expression of myc-tagged ligase IV protein and three independent clones expanded for each construct.

Immunoprecipitation and western blot analysis

CHO XR-1 cells expressing myc-tagged DNA ligase IV proteins were lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EDTA, 1 mM DTT, 1% NP-40, 1 mM Na3VO4 and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min at 4 °C with rocking. To detect myc- and EGFP-tagged proteins, 50–100 μg of whole cell extract (WCE) were resolved using SDS–PAGE and transferred to a nitrocellulose membrane for immunoblotting. c-myc (9E10) mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) was used to examine expression of myc-tagged proteins. GFP (ab290) rabbit polyclonal antibody (Abcam limited, Cambridge, UK) was used for analysis of EGFP-tagged proteins. 800–1000 μg of WCE were immunoprecipitated using c-myc antibodies and protein G Sepharose beads (Amersham Pharmacia Biotech AB). Membranes were probed with primary antibody over-night. Blots were probed with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dako A/S, Denmark) and developed using the Renaissance chemiluminescence system (NEN™ Life Science products).

Analysis of CHO-expressed proteins

CHO XR-1 cells expressing myc-tagged DNA ligase IV proteins were transiently transfected with pEGFP-C3 or pEGFPPhXRCC4 vectors and lysed 48 h later as described earlier. Immunoprecipitated myc-tagged proteins were washed twice with lysis buffer and incubated for 15 min at room temperature in adenylation buffer (60 mM Tris–HCl, pH 8, 10 mM MgCl2, 0.1 mg/ml BSA) supplemented with 5 mM Na pyrophosphate. Sepharose beads were washed twice in adenylation buffer and the adenylation assays were carried out in 30 μl of adenylation buffer supplemented with 5 mM DTT and 10 μCi of [α-32P] ATP at 800 mCi/mmol (ICN) for 45 min at room temperature. Beads were then washed twice with adenylation buffer and proteins separated by SDS–PAGE. The gel was fixed, dried and analysed on a STORM phosphorimagor (Molecular Dynamics).

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Intracellular staining of transfecteds for myc-ligase IV proteins was carried out as described previously (22). Briefly, transfected cells were washed twice in PBS, fixed in 3.7% paraformaldehyde and permeabilized in Triton buffer. Anti-myc monoclonal antibody and the secondary anti-mouse FITC (Sigma) were used at dilutions of 1:200. For visualization of EFGP-tagged proteins, cells were washed, fixed and permeabilized, as described earlier.

Expression and purification of DNA ligase IV/XRCC4 complexes in insect cells
DNA ligase IV and XRCC4 were co-expressed in the Bac-To-Bac<sup>TM</sup> Baculovirus expression system (Gibco-BRL) and purified to near homogeneity on metal-chelate TALON affinity resin as described previously (18). Purified complexes were concentrated on 0.5 ml Vivaspin 500 columns (Sartorius, 100 kDa cut-off size) and stored frozen at −80°C. The concentration step also served to remove any uncomplexed XRCC4 and DNA ligase IV proteins. The purity of all preparations was assessed using 9% SDS–PAGE.

Ligation and adenylation assays
Ligation and adenylation assays were carried out as described previously (16,19).

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