Folding and Assembly of Type X Collagen Mutants That Cause Metaphyseal Chondrodysplasia-type Schmid

EVIDENCE FOR CO-ASSEMBLY OF THE MUTANT AND WILD-TYPE CHAINS AND BINDING TO MOLECULAR CHAPERONES

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Type X collagen is a small non-fibrillar collagen (MW 59,000) synthesized exclusively in the epiphyseal growth plate during chondrocyte hypertrophy (1). The overall domain structure of type X collagen is similar to the fibrillar procollagens in that there are three distinct domains: a short amino-terminal domain of 161 amino acids (2). However, unlike the fibrillar procollagens, the propeptides are not cleaved following secretion into the extracellular matrix and are thought to play a role in assembly of the individual molecules into higher order structures (3).

Mutations within the COL10A1 gene result in Schmid metaphyseal chondrodysplasia (SMCD), an autosomal dominant disorder of the osseous skeleton. The majority of these mutations map to the NC1 domain and include amino acid substitutions, nonsense mutations, and deletions (4). The only exceptions so far found are at the junction of the signal sequence and the NC2 domain (5) that may effect signal peptide cleavage. Significantly no mutations within the triple helical domain have been identified. Mutation within the NC1 domain has been proposed to effect the initial stages in the folding and assembly of type X collagen which are thought to occur in a similar fashion to the fibrillar procollagens (6). Once fully translocated into the ER lumen, the constituent chains of the trimer associate via their carboxyl-terminal globular domains allowing nucleation of the triple helix to occur at the carboxyl-terminal end (7) with subsequent propagation of the helix in a zipper-like fashion from the COOH- to the NH2-terminal direction (8). Therefore mutations in the NC1 domain would be predicted to disrupt the initial association of type X chains and prevent nucleation of the triple helix.

This hypothesis has been supported by studies on the in vitro transcription and translation of mutant and wild-type cDNAs within a cell-free system (9). The ability of the NC1 domain to associate and form trimers was assayed by the resistance of the trimer to denaturation by SDS. The wild-type trimer was shown to be particularly resistant to dissociation by SDS, a property not shared by the mutant chains. These results were taken to indicate that the mutant chains did not assemble and therefore that the effect of the mutation was to prevent correct folding of the mutant chain leading to its retention within the ER and subsequent degradation. Thus attempts to express mutant type X chains in cells grown in culture led to very poor levels of expression with little or no secretion of the mutant type X chains (10).

Lack of secretion of mutant chains would result in a reduction of the extracellular type X available for assembly. Thus, the current model for the mechanism of SMCD is haploinsufficiency. Here the phenotype would be explained simply by a 50% reduction in the level of type X secreted rather than a direct effect of the mutant chains on the assembly of the wild-type chains. This model is supported by the failure to detect, in one SMCD patient, any mutant type X protein in the extracellular matrix due to the degradation of the mutant transcript that contains a premature stop codon (11). This is corroborated by analysis of transgenic mice that are null alleles for type X that show some of the phenotypic changes associated with

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The abbreviations used are: NC2, non-collagenous amino-terminal; SMCD, Schmid metaphyseal chondrodysplasia; NC1, non-collagenous carboxy-terminal; ER, endoplasmic reticulum; SP, secreted, semi-permeabilized HT1080 fibroblasts; DSP, dithiobis(succinimidyl propionate); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; C-propeptide, carboxyl-terminal propeptide; BiP, immunoglobulin heavy chain-binding protein.
SMCD (12). However, analogous transgenic null allele mice show no significant phenotypic change (13).

Other evidence, however, raises the possibility that haploinsufficiency may not be the only mechanism underlying SMCD. Transgenic mice expressing chicken type X with a truncated helix show skeletal abnormalities (14), pointing toward a dominant negative interference of the function of type X in the extracellular matrix. Trace amounts of heterotrimer could be detected during cell-free co-expression of wild-type and some point mutants (9) suggesting some co-assembly of these chains could occur. Significantly, the distribution of mutations within the NC1 domain are not random but clustered into three distinct regions (4) which is counterintuitive to a general folding defect in type X unless these regions alone are critical for assembly.

The expression studies of mutant and wild-type chains using cell-free systems did not fully reconstitute the initial stages of collagen assembly (9, 10). We have previously demonstrated that the complete folding pathway of type X collagen can be reconstituted in semi-permeabilized cells (SP cells) (15). This system was used here to address the question of whether mutant type X collagen chains can initiate triple helix formation within the ER lumen. We expressed type X containing SMCD mutations within each of the three clusters; Y598D (16), G617K (17), and W651R (18), and analyzed their effects on folding, assembly, and triple helix formation (15). In contrast to previous studies, we demonstrate that all three mutants can individually associate to form homotrimers to form thermally stable triple helices. We also show that heterotrimers can form between the wild-type and mutant type X and that the pattern of binding of the wild-type and mutant chains to molecular chaperones is identical.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Plasminoids and Mutagenesis—Recombinant bovine type X maintained in pSKBX has been previously described (15). Site-directed mutagenesis was carried out using the Quickchange kit (Stratagene Ltd., Cambridge, United Kingdom) using the accompanying protocol. Using pSKBX as a template, the following complementary primers were used to generate point mutations (underlined) in the NC1 domain of bovine type X (amino acids are numbered from the translation start site of human pre-a1(X)): 5′-GATTCCAGGATATAATGATCTTGTACCAAC-3′ for Tyr598→Asp (pSHM14); 5′-AGACACGGTTAGGCTCCTCTG-3′ for Arg (pSHM15); and 5′-GTAGGCTCCCTTCTC-3′ for Lys (pS2). HA-tagged and Myc-tagged wild-type and mutant type X molecules were generated by PCR overlap extension using the principles outlined by Horton (19). PCR reactions (100 μl) comprised template DNA (250 ng), oligonucleotide primers (100 pmol each) in 20 mM Tris-HCl, pH 8.8, ly 300 base pairs were generated using a 5′-overhang (England Biolabs, Beverly, MA) and the NC1 domain are not random but clustered into three distinct regions (4) which is counterintuitive to a general folding defect in type X unless these regions alone are critical for assembly.

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In Vitro Translation in Semi-permeabilized Human Fibroblasts (HT1080)—Cultured HT1080 fibroblasts (ATCC-CCL121, American Type Culture Collection, Rockville, MD) were incubated in the absence of the detergent digitonin (Calbiochem, Nottingham, UK) at a final concentration of 40 μg/ml using a modification of the method of Plutner et al. (21) as described in Ref. 22. RNA was translated using a rabbit reticulocyte lysate (Flexi-Lyase, Promega) at 30 °C in the absence of exogenous dithiothreitol. The translation reaction (25 μl) contained 17.5 μl of reticulocyte lysate, 0.5 μl of 1 mM amino acids (minus methionine), 0.5 mM MgCl₂, 1.5 μl of 0.1 M Tris-HCl, pH 7.4, and 20 μl of 1 M KCl and incubated prior to endoglycosidase H digestion, chemical cross-linking, proteolysis, and Endo H Digestion—Products of translation were digested with endoglycosidase H as described previously (23).

Chemical Cross-linking—After translation cell pellets were resuspended in 100 μl of KHM buffer and dithiothreitol (succinimidyl propionate) (DSP, Sigma) was added to 1 mM final concentration from a 100 mM stock in dimethyl sulfoxide, followed by incubation at room temperature for 10 min. The cross-linker was quenched by addition of glycine to 40 mM, and incubating for a further 5 min.

Prior to immunoprecipitation the cross-linked samples were denatured by adding SDS to 0.5% (w/v) final concentration and heating to 100 °C for 1 min. Immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% (w/v) gelatin, 0.10% (v/v) Nonidet P-40) was then added to 2 ml and samples were precleared by incubation with 40 μl of protein A-Sepharose (10% (v/v) preincubated in NET-gel buffer containing 5% bovine serum albumin) (Zymed Laboratories Inc., San Francisco, CA) for 40 min at 4 °C. After removal of protein A-Sepharose-bound material by centrifugation, the appropriate anti-serum was added at a dilution of 1:500 along with 40 μl of protein A-Sepharose and samples incubated for 3 h at 4 °C. The goat anti-BiP antibody was supplied by Santa Cruz Biotechnology, Santa Cruz, CA. The mouse anti-Hsp 47 monoclonal antibody was from Stressgen Biotechnologies Corp., Victoria, Canada. The rabbit anti-protein disulfide isomerase polyclonal antibody was raised to purified bovine protein disulfide isomerase as described previously (24). After incubation, pelleted complexes were washed three times with 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100.

Sequential Immunoprecipitations—After translation cell pellets were solubilized in 1 ml of immunoprecipitation buffer and samples precleared by incubation with 40 μl of 10% (v/v) protein A-Sepharose. After removal of protein A-Sepharose-bound material by centrifugation, mouse anti-Myc monoclonal antibody (9E10) (Calbiochem, Nottingham, UK) was added to a 1:500 dilution along with 40 μl of 10% (v/v) protein A-Sepharose. After incubation, pelleted complexes were washed three times with 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100. The material immunoprecipitated with the anti-Myc antibody was eluted from the beads by addition of SDS to 1% (w/v) in a volume of 100 μl and heating to 100 °C. A small volume (10 μl) was taken for gel analysis while the rest of the sample was diluted to 1 ml in immunoprecipitation buffer. Any HA-tagged proteins were then immunoprecipitated with a rabbit anti-HA (12CA5) antibody diluted to 1:500 (Boehringer Mannheim, Lewes, Sussex, UK). Sample supernatants were removed and cell pellets were re-suspended in CTB buffer (50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 10 mM EDTA, 0.5% (v/v) Triton X-100). Chemotropsin and trypsin (Sigma) were added to 250 and 100 μg/ml final concentrations and samples incubated at room temperature for 5 min. Further digestion was stopped by the addition of soybean trypsin inhibitor to a final concentration of 5 mg/ml and 2.5 volume of boiling SDS-PAGE loading buffer.
Co-assembly of Wild-type and Mutant Type X Collagen

 containing 5% (v/v) 2-mercaptoethanol, and the samples were boiled for 5 min.

Thermal Denaturation—Pelleted SP cells were resuspended in CT/T buffer and 16-µl aliquots were placed in a DNA thermal cycler. A stepwise temperature gradient was set up from 25 to 47 °C with the temperatures being held for 2 min at each temperature interval. For a given temperature at the end of 2 min the sample was assayed with chymotrypsin/trypsin for 2 min as described above.

SDS-PAGE—Samples were prepared for electrophoresis by mixing with an equal volume 2 times SDS-loading buffer (0.125 M Tris-HCl, pH 6.8, 2% (v/v) SDS, 20% (v/v) glycerol, and 0.004% (v/v) bromphenol blue). When resistance to denaturation by heating in the presence of SDS was assayed a lower final concentration of SDS was used (0.5% w/v). Samples were reduced by addition of 5% (v/v) 2-mercaptoethanol. The samples were boiled for 5 min before electrophoresis, unless otherwise stated. After electrophoresis, dried gels were exposed to Kodak X-Omat AR film or analyzed on a Fuji BAS 2000TR PhosphorImager.

RESULTS

Analysis of the Ability of Wild-type and SMCD Mutants to Form Trimmers Resistant to Denaturation with SDS—Previous experiments carried out to determine the ability of wild-type and mutant type X chains to form homotrimers involved expressing the RNA transcripts coding for these proteins in a cell-free system. The ability to assemble was assessed by the formation of a trimer that was resistant to denaturation after heating to 60 °C in presence of 2% (w/v) SDS and 2 M urea (9, 10). Under these conditions only the wild type was able to form a resistant trimer suggesting that the effect of the mutation was to prevent trimer formation. We wanted to determine whether the mutant chains were able to form trimers at all and whether the trimers formed would be more stable under milder denaturing conditions.

For our experiments we used the bovine cDNA clone, the NC1 domain of which shares over 90% identity with the human NC1 domain. All the amino acids that have been identified as targets for mutations giving rise to SMCD are identical. Thus we constructed three analogous SMCD point mutations: Y598D which lies within a hydrophobic region of the NC1 domain predicted to be critical for trimer formation (25); G617K which disrupts the potential N-glycosylation site (Asn-Gly-Thr) in the NC1 domain (17); and W651R which lies in the third cluster of mutations in the NC1 domain (18). The cDNAs clones coding for these mutants were transcribed in vitro and translated in a reticulocyte lysate supplemented with SP cells that been shown to reconstitute the initial folding and assembly of fibrillar collagens and type X collagen (15, 26). The ability of the various mutants to assemble to form trimers was assayed by their resistance to denaturation by heating in the presence of 0.5% (w/v) SDS. As can be seen (Fig. 1) the wild-type and mutant chains were all able to assemble to form trimers which were resistant to denaturation when heated up to 60 °C but which were fully denatured at 100 °C. The N617K mutant formed these resistant trimers less efficiently than either the wild-type or other mutant chains, but the fact that any resistant trimers are formed demonstrates that the mutated NC1 domains can associate with themselves to form homotrimers. We confirmed previous results (9) demonstrating that the trimerization of the wild-type and mutant chains to form SDS-resistant molecules was due to an association at the NC1 domain by carrying out collagenase digestion (results not shown). At higher SDS concentrations all the mutant chains were denatured when heated to 60 °C whereas the wild-type chain was not (results not shown), confirming the previous results obtained with the human type X SMCD mutant chains (9).

The fact that the N617K mutant forms a trimer resistant to denaturation under these conditions less efficiently than the other chains could be due to this mutation abolishing the consensus sequence for glycosylation. This in turn could effect the solubility of the monomer and therefore the assembled trimer.

As there is some confusion as to whether this glycosylation site is actually utilized in type X collagen we carried out endoglycosidase H digestion of the wild-type and mutant chains following translation in the presence of SP cells to determine the glycosylation status. As a control we translated tissue-type plasminogen activator which contains three sites for N-link glycosylation (27). Clearly the tissue-type plasminogen activator translation product is susceptible to digestion with endoglycosidase H (Fig. 2, lanes 1 and 2) whereas there is no difference in mobility of the wild-type or N617K translation products after digestion (Fig. 2, lanes 3 and 4, lanes 5 and 6). There is also no difference in mobility between the wild-type and N617K mutant which would be expected if the glycosylation site was occupied. These results clearly demonstrate that this site is not occupied in type X collagen. The actual sequence in type X collagen has a proline residue following the glycosylation site. A proline in this position has previously been shown to inhibit glycosylation (28) providing a reason for the lack of occupancy of this site in type X collagen.

Can Mutant Chains Assemble to Form Correctly Folded Triple Helical Molecules?—Once we had shown that the type X chains containing point mutations associated with SMCD could associate to form homotrimers, we then wanted to determine whether the triple helical domains within these chains would fold correctly. Translation was carried out in the presence of SP cells and ascorbate to ensure hydroxylation of proline residues and the formation of a triple helix was assayed by resistance to digestion by a combination of trypsin and chymotrypsin. The results (Fig. 3A) show that protease-resistant fragments corresponding to the collagenous domain of type X were generated after treatment of both wild-type and mutant chain with proteases. The efficiency of triple helix formation was determined by quantification of the translation products before and after protease treatment. The results (average over three experiments) illustrated that the wild-type chain folded efficiently (94%) whereas the mutant chains folded less efficiently than the wild-type (Y598D, 55%; N617K, 63%; W651R, 70%). The thermal stability of the triple helical domains formed by the wild-type and mutant chains was also assayed (Fig. 3B). The denaturation temperatures of the triple helix formed were very similar indicating that the extent of hydroxylation of the type X
collagen is not influenced by these mutations in the NC1 domain. Thus the mutant chains are able to associate to form trimers and once these trimers have formed they can fold correctly to form a stable triple helix.

Can the Mutant Chains Co-assemble with Wild-type Chains?—Having established that mutant chains can form homotrimers we then went on to determine if the mutant chains could co-assemble with wild-type chains to form heterotrimers. The approach we took was to add one epitope tag (HA) to the wild-type chain and a different epitope tag (Myc) to a mutant chain (for these experiments we used the Y598D mutant). After co-translation of the two chains in the presence of SP cells we first immunoprecipitated the mutant chains with an anti-Myc antibody. Any wild-type chains that co-assembled with the mutant chains would also be immunoprecipitated under these conditions. The co-assembled wild-type chains could then be identified by first denaturing the anti-Myc immunoprecipitate and then immunoprecipitating with anti-HA. As a positive control for co-assembly, we tagged wild-type chains with Myc and co-translated Myc-tagged along with HA-tagged wild-type chains. We first established that the antibodies could specifically recognize the variously tagged chains by translating them individually. The wild-type chain tagged with the HA epitope was not immunoprecipitated with anti-Myc and consequently no radiolabeled product was immunoprecipitated with anti-HA from the anti-Myc immunoprecipitate (Fig. 4, lanes 1 and 2). In contrast, the Y598D chain tagged with the Myc epitope was immunoprecipitated with anti-Myc but no radiolabeled protein was immunoprecipitated with anti-HA from the anti-Myc immunoprecipitate (Fig. 4, lanes 3 and 4). These results demonstrate that there is no nonspecific immunoprecipitation of chains using this sequential immunoprecipitation approach. When the wild-type chain tagged with the HA epitope was co-translated with either the wild-type chain or the Y598D chain tagged with the Myc epitope, radiolabeled material was present in the anti-Myc immunoprecipitate (Fig. 4, lanes 5 and 7). Between 5 and 10% of this material was subsequently immunoprecipitated by anti-HA (Fig. 4, lanes 6 and 8) demonstrating that the HA-tagged wild-type or the HA-tagged Y598D chains co-assembled with the Myc-tagged wild-type chains. We carried out a similar experiment by first immunoprecipitating with the anti-Myc antibody, carrying out a second immunoprecipitation with the anti-HA antibody, and obtained similar results (results not shown). These results demonstrate that within the environment of the ER both wild-type and chains containing SMCD mutations can co-assemble to form heterotrimers.

Interaction of Type X Collagen Chains with Molecular Chaperones—The main conclusions that could be drawn from the preceding experiments was that both wild-type and mutant chains can assemble and fold to form homo- or heterotrimers. The trimers formed, however, differed in their sensitivity to heat denaturation in the presence of SDS which would suggest that the NC1 domain formed from mutant chains adopted a more open conformation than the NC1 domain formed from wild-type chains. One consequence of this change in conformation could be a more stable interaction with chaperone proteins within the ER, which in turn would lead to retention of the mutant protein within the cell. Alternatively mutant chains could interact with a different set of chaperones than the wild-type chains resulting in differential retention. To assess this point we determined whether there were any differences in the types of molecular chaperones that the wild-type or mutant chains interacted with in our SP cells. We translated both the wild-type and mutant chains individually and after isolation of the cells from the translation mixture cross-linked translated chains to interacting proteins using the bifunctional cross-linking reagent DSP. Essentially we obtained the same results irrespective of the type of chain we translated (Fig. 5). We have previously shown that the two main proteins that interact with type X are protein disulfide isomerase (Fig. 5, lanes 1–4) and Hsp 47 (Fig. 5, lanes 9–12). Previous work on mutation within the C-propeptide of fibrillar procollagens has shown that mutations that disrupt folding of the monomer result in an interaction of the misfolded chain with BiP (29). We could not detect any interaction of either the wild-type or mutant chains with BiP using this approach even though we have previously shown that the anti-BiP antibody can immunoprecipitate BiP following this cross-linking treatment (30). Thus our results do not suggest any significant differences in the way in which the main collagen binding chaperones interact with wild-type and mutant chains.

DISCUSSION

The main objective of this study was to ascertain whether the phenotype underlying SMCD, which has been suggested to be due to a reduction in the level of secretion of mutant chains, could be explained by an intracellular folding and transport defect. The previous studies on the synthesis and folding of type X collagen chains containing point mutations associated with SMCD clearly demonstrated a difference in the resistance of the protein to dissociation by heating in the presence of SDS and urea (9, 10). Here we show that this observation does not mean that the mutant chains cannot assemble to form trimers, only that the trimers once formed are more susceptible to dissociation than the wild-type under the conditions of this assay. Indeed the mutant chains were not only able to form homotrimers but were capable of nucleating triple helix formation to form a thermally stable triple helix. Interestingly the mutant chains were also able to co-assemble with the wild-type chains to form heterotrimers. These conclusions do not rely on the formation of NC1 domain trimers resistant to heating in the presence of SDS. Thus by three independent assays we have shown that point mutations associated with SMCD do not prevent association of the mutant chains with themselves or with wild-type chains. These results prove that the molecular mechanism underlying SMCD cannot be exclusively explained by haploinsufficiency as the mutant chains can co-assemble with wild-type chains. There is, however, at least one case
where a 50% reduction of the level of expression of type X collagen leads to a SMCD phenotype (11).

Clearly the mutations do alter the conformation of any homotrimers formed and would be predicted to have varying effects on the conformation of heterotrimers. This could lead to retention of trimers containing mutant chains within the ER with the consequence of a reduction in the level of type X chains secreted. Such a mechanism of ER “quality control” prevents the secretion of incompletely assembled or malfolded proteins and can be mediated either by glycoprotein-specific chaperones such as calnexin and calreticulin (31), by interaction with BiP or other ER-resident proteins (32), or by a thiol-mediated proc-ess (33). Attempts to evaluate whether homotrimers containing mutant chains could be secreted from intact cells have been hampered by their low levels of expression (10). This low level of secretion in itself could be due to an efficient mechanism of retention of mutant chains leading to an increase in the intracellular degradation of the mutant homotrimers. No attempts to express both wild-type and mutant chains in the same cell have yet been reported and it would indeed be interesting to determine if heterotrimers of mutant and wild-type chains could be secreted. In this scenario the SMCD phenotype would be explained not by a lack of folding and assembly of the mutant chains but rather by a decrease in their stability with the overall result that fewer type X chains would be secreted and be available for assembly.

The alternative explanation to intracellular degradation of trimers containing mutant chains is that these molecules are secreted normally but interfere with the normal function of type X collagen outside the cell. The most persuasive argument for such a mechanism to explain the SMCD phenotype arises...
from the fact that only these specific mutations give rise to SMCD. Other mutations within the NC1 domain that could effect folding of the monomer or the association of the monomers to form trimers would also be degraded and lead to a reduction in the levels of type X collagen secreted and should give rise to a SMCD phenotype. Thus the main characteristic of at least the point mutations studied here which cause SMCD is that they do not cause a general folding defect. This would argue that the effect of the point mutation is on either the assembly of the protein outside the cell or on an interaction with other extracellular matrix proteins as has been suggested previously (4). The crucial experiment to verify such a mechanism would be the isolation of type X molecules containing mutant chains in the extracellular matrix of affected individuals.

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REFERENCES
1. Schmid, T. M., and Linseismayer, T. F. (1983) J. Biol. Chem. 258, 9504–9509
2. Thomas, J. T., Kwan, A. P. L., Grant, M. E., and Boot-Handford, R. P. (1991) Biochem. J. 273, 141–148
3. Kwan, A. P. L., Chapman, J. A., and Grant, M. E. (1991) J. Cell Biol. 114, 597–604
4. Wallis, G. A., Rash, B., Sykes, B., Bonaventure, J., Maroteaux, P., Zabel, B., Wynnmedavies, R., Grant, M. E., and Boothandford, R. P. (1996) J. Med. Genet. 33, 450–457
5. Ikegawa, S., Nakamura, K., Nagano, A., Haga, N., and Nakamura, Y. (1997) Hum. Mutat. 9, 131–135
6. McLaughlin, S. H., and Bulleid, N. J. (1998) Matrix Biol. 16, 369–377
7. Bulleid, N. J., Dailey, J. A., and Lees, J. F. (1997) EMBO J. 16, 6694–6701
8. Engel, J., and Prockop, D. J. (1991) Annu. Rev. Biophys. Biophys. Chem. 20, 137–152
9. Chan, D., Cole, W. G., Rogers, J. G., and Bateman, J. F. (1995) J. Biol. Chem. 270, 4558–4562
10. Chan, D., Weng, Y. M., Hocking, A. M., Gelub, S., McQuillan, D. J., and Bateman, J. F. (1996) J. Biol. Chem. 271, 13566–13572
11. Chan, D., Weng, Y. M., Graham, H. K., Silence, D. O., and Bateman, J. F. (1999) J. Clin. Invest. 101, 1490–1499
12. Kwan, M. K. M., Zhou, S., Cowan, S. K., Kong, R. Y. C., Pfordte, T., Olsen, B. R., Silence, D. O., Tam, P. P. L., and Cheah, K. S. E. (1997) J. Cell Biol. 136, 459–471
13. Rosati, R., Horan, G. S. B., Pinero, G. J., Garofalo, S., Keene, D. R., Horton, W. A., Vuerio, E., Derrbrugge, B., and Behringer, R. R. (1994) Nat. Genet. 8, 129–135
14. Jacenko, O., Luvvalle, P. A., and Olsen, B. R. (1995) Nature 365, 56–61
15. McLaughlin, S. H., and Bulleid, N. J. (1998) Biochem. J. 331, 795–800
16. Wallis, G. A., Rash, B., Sweetman, W. A., Thomas, J. T., Super, M., Evans, G., Grant, M. E., and Boothandford, R. P. (1994) Am. J. Hum. Genet. 54, 169–178
17. Bonaventure, J., Chaminade, F., and Maroteaux, P. (1995) Hum. Genet. 96, 58–64
18. Pokharel, R. K., Alimsardjono, H., Uno, K., Fuji, S., Shiba, R., and Matsuo, M. (1995) Biochem. Biophys. Res. Commun. 217, 1157–1162
19. Horton, R. M. (1993) in Methods in Molecular Biology (White, B. A., ed) Vol. 15, pp. 251–261, Humana Press Inc., Totowa, NJ
20. Gurevich, V. V., Pokrovskaya, I. D., Obukhova, T. A., and Zozulya, S. A. (1991) Anal. Biochem. 195, 207–213
21. Plutner, H., Davidsen, H. W., Saraste, J., and Balch, W. E. (1992) J. Cell Biol. 119, 1097–1116
22. Wilson, R., Allen, A. J., Fowler, J., Brooksman, J. L., High, S., and Bulleid, N. J. (1995) Biochem. J. 307, 679–687
23. Bulleid, N. J., and Freedman, R. B. (1988) Biochem. J. 254, 805–810
24. John, D. C. A., Grant, M. E. G., and Bulleid, N. J., (1993) EMBO J. 12, 1587–1595
25. Brass, A., Kadar, K. E., Thomas, J. T., Grant, M. E., and Boothandford, R. P. (1992) FEBS Lett. 303, 126–128
26. Bulleid, N. J., and Lees, J. F. (1996) Biochem. J. 317, 195–200
27. Allen, S., Naim, H. Y., and Bulleid, N. J. (1996) J. Biol. Chem. 270, 4797–4804
28. Meilquiat, J. L., Kasturi, L., Spitalnik, S. L., and ShakinEnhleman, S. H. (1998) Biochemistry 37, 6833–6837
29. Chessler, S. D., and Byers, P. H. (1992) J. Biol. Chem. 267, 7751–7757
30. Wilson, R., Lees, J. F., and Bulleid, N. J. (1998) J. Biol. Chem. 273, 9063–9064
31. Tatu, U., and Helenius, A. (1997) J. Cell Biol. 136, 555–565
32. Fourie, A. M., Sambrook, J. F., and Getting, M. H. J. (1994) J. Biol. Chem. 269, 30470–30478
33. Ronay, P., Sparvoli, A., Fagidi, C., Fassina, G., and Sitia, R. (1996) EMBO J. 15, 2077–2085