Detection of an appropriate kinase activity in branchial arches I and II that coincides with peak expression of the Treacher Collins syndrome gene product, treacle

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Treacher Collins syndrome (TCS) is an autosomal dominant craniofacial disorder involving the mid and lower face and, in particular, the tissues affected arise solely from embryonic branchial arches I and II. TCOF1, the gene involved in TCS, has been cloned and although the function of the encoded protein, treacle, has not yet been established, it exhibits peak expression in the branchial arches. Treacle contains a series of repeating units of acidic and basic residues, which are predicted to contain putative casein kinase II (CKII) and protein kinase C (PKC) phosphorylation site motifs. In addition, treacle has weak homology to two phosphorylation-dependent nucleolar proteins, which shuttle between the cytoplasm and nucleolus. Based on these observations, phosphorylation of treacle may be important for its function. In this study, GST–treacle fusion peptides were constructed using particular TCOF1 exons that contained potential CKII and PKC phosphorylation sites. These were used as substrates in in vitro kinase assays and showed that treacle fusion peptides can be phosphorylated by the appropriate kinases. Furthermore, using tissue extracts we have demonstrated that in avian embryonic branchial arches I and II there is a kinase activity that can phosphorylate treacle peptides that is consistent with CKII site recognition. This activity coincides with the reported high expression of treacle in these tissues at early developmental stages and declines later in development.

INTRODUCTION

Treacher Collins syndrome (TCS), an autosomal dominant disorder of craniofacial development, occurs with an incidence of 1/50 000 live births (1,2). While 40% of TCS cases have a previous family history, 60% of cases possibly arise as a result of a de novo mutation (3). The clinical features of TCS are generally symmetrical (4) and include: (i) abnormalities of the external ears, narrowing of the external ear canals and malformation of the middle ear ossicles, which may lead to conductive hearing loss (5); (ii) lateral downward sloping of the palpebral fissures with colobomas of the lower eyelids and lack of lower eyelashes; (iii) hypoplasia of the mandible and zygomatic complex and cleft palate (1,6). TCS displays high penetrance, with expression of the clinical phenotype and severity of the disorder showing inter- and intrafamilial variability (7).

The TCS gene, TCOF1, was identified via positional cloning (8–11) and found to encode a low complexity, serine/alanine-rich protein which was named treacle (12,13). Analysis of this gene in TCS affected individuals identified over 50 disease-causing mutations that were spread throughout TCOF1, with the majority of the mutations introducing a premature termination codon (12–15). Although the TCOF1 gene has been cloned, the precise function and biochemical nature of the encoded protein has not been determined. There are 10 repeating units containing clusters of acidic amino acids that are separated by basic amino acid residues within treacle (7). Each repeating unit is confined to a single exon and comparison against pattern databases predicts that these repeat units contain putative casein kinase II (CKII) and protein kinase C (PKC) phosphorylation site motifs (7). An additional feature of treacle is the identification of potential nuclear localization signals (NLSs) near the C-terminus (7). Recent studies have demonstrated that these NLSs enable treacle to be targeted to the nucleolus (16,17).

Database comparisons have revealed weak homology between treacle and two phosphorylation-dependent nucleolar phosphoproteins, Xenopus laevis nucleolar phosphoprotein and rat nucleolar phosphoprotein 140 (Nopp140) (7,18,19). These nucleolar phosphoproteins shuttle between the nucleolus and cytoplasm, suggesting a role as chaperones in protein transport (7,19). All three proteins appear to be low complexity proteins, with a majority of the same five amino acids (i.e. serine, alanine, lysine, proline and glutamic acid). Furthermore, the sequence similarity between treacle and the nucleolar phosphoproteins is greatest at the repeating motifs, where the potential sites for CKII phosphorylation are predicted. The similarity of treacle to nucleolar phosphoproteins such as Nopp140 suggests that treacle may function in protein import or export by shuttling between the nucleolus and cytoplasm. This hypothesis is supported by recent work which showed that mutations in TCOF1 cause the resulting truncated protein to become mislocalized within the cell (16).

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On the basis of the TCS phenotype, treacle must play a fundamental role in embryonic development. The craniofacial defects observed in TCS affect tissues derived from branchial arches I and II, which form during the first 4 weeks of human development (20). Since neural crest cells contribute significantly to formation of the branchial arches, dysmorphogenesis of the lower face may therefore be a consequence of defective neural crest cell migration from the hindbrain to the branchial arches during early embryogenesis (21,22) and/or neural crest cell survival, proliferation, differentiation or metabolism. Hence, knowledge of the processes involved in development of the branchial arches or their derivatives may lead to identification of a function for the treacle protein. Further analysis was achieved through isolation of the murine homologue of the gene involved in TCS, tcof1. (15). tcof1 has a slightly smaller open reading frame than human TCOF1, but it also encodes a low complexity, serine/alanine-rich protein (15). Observations from expression analysis in the mouse support the suggestion that the TCS gene is involved in development of the craniofacial complex during embryogenesis (15), since it is highly expressed in the early branchial arches, while also being expressed at lower concentrations at other sites and stages.

In our current investigation, \textit{in vitro} kinase assays were used to demonstrate whether glutathione S-transferase (GST)–treacle fusion peptides containing potential CKII and PKC phosphorylation motifs could be phosphorylated by the appropriate kinases. Subsequent experiments using crude tissue extracts derived from various avian tissues, including branchial arches, showed that a kinase activity that could use a specific treacle peptide as a substrate was expressed in the appropriate temporal pattern (i.e. during early embryonic development). Thus, this kinase activity broadly coincides with peak expression of treacle (i.e. at early stages in branchial arches I and II), which is consistent with phosphorylation playing a role in the biology of treacle.

\section*{RESULTS}

\subsection*{Construction of GST–treacle fusion peptides}

Three GST–treacle fusion peptides were constructed that contained either \textit{TCOF1} exon 9, exon 14 or exon 22 (Fig. 1). These fusion peptides represented the possible kinase targets predicted by pattern database analysis (i.e. CKII/PKC target, exon 9; PKC/alternative kinase target, exon 14; no kinase target, exon 22). The exon 14 GST–treacle fusion peptide was estimated to be 32.5 kDa, the exon 22–treacle fusion peptide was \textsim 34.5 kDa, whilst the exon 9 GST–treacle fusion peptide was \textsim 35.5 kDa. The GST–treacle fusion peptides were expressed in bacterial cultures following IPTG induction and subsequently purified from the bacterial lysates via affinity chromatography. Results from SDS–PAGE (Fig. 2) show that the GST–treacle fusion peptides were of the correct size. Following staining with Coomassie brilliant blue dye, two bands of the correct molecular size (i.e. 35.5 kDa), with a consistent band of smaller size also apparent. Inclusion of protease inhibitors in the solubilization buffer made no difference to the two bands representing the exon 9 fusion peptide (data not shown).

\subsection*{In vitro kinase assays using purified kinases with GST–treacle fusion peptides}

\textit{In vitro} kinase assays were used to examine whether treacle peptides could be phosphorylated by commercially available kinases. \textit{In vitro} phosphorylation of the GST–treacle fusion peptides in the presence of purified CKII and PKC was demonstrated following SDS–PAGE analysis of the kinase assay reaction products. There was no phosphorylation of the GST protein by either CKII or PKC and this is consistent with the GST protein not containing a kinase recognition motif. No CKII kinase phosphorylation sites were detected in the exon 14 GST–treacle fusion peptide and the two characteristic bands at \textsim 32.5 kDa, the exon 22 GST–treacle fusion peptide at \textsim 34.5 kDa and the two characteristic bands at \textsim 35.5 kDa correspond to the exon 9 GST–treacle fusion peptide.

\begin{figure}[h]
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\caption{Schematic diagram of the GST–treacle fusion peptide constructs. The constructs generated for this study were produced after cloning the desired \textit{TCOF1} exons followed by subcloning them into the GST gene fusion vector pGEX2T. (A) GST–treacle fusion peptide containing exon 9; (B) GST–treacle fusion peptide containing exon 14; (C) GST–treacle fusion peptide containing exon 22.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure2.png}
\caption{Purified GST–treacle fusion peptides. Purified GST–treacle fusion peptides (indicated by arrows) were detected with Coomassie brilliant blue dye following SDS–PAGE. A band representing the GST protein was detected at \textsim 27.5 kDa. The remaining bands correspond to the exon 14 GST–treacle fusion peptide at \textsim 32.5 kDa, the exon 22 GST–treacle fusion peptide at \textsim 34.5 kDa and the two characteristic bands at \textsim 35.5 kDa correspond to the exon 9 GST–treacle fusion peptide.}
\end{figure}
peptides containing exon 14 and exon 9 were found to be substrates for PKC activity, whilst very weak phosphorylation was detected in the lane containing the exon 22 GST–treacle fusion peptide (Fig. 3B).

**In vitro kinase assays using crude protein extracts of avian tissues with GST–treacle fusion peptides**

Branchial arches I and II from embryonic day (E) 3.5 quail embryos were isolated as a potential source of kinase activity that could phosphorylate treacle peptides. SDS–PAGE analysis of the kinase assay reaction products following affinity purification indicated the presence of a kinase active on both bands of the exon 9 GST–treacle fusion peptide in the branchial arch extracts (Fig. 4). No phosphorylation of the exon 14 or exon 22 GST–treacle fusion peptides was detected. Further analysis to investigate whether treacle peptides could act as kinase substrates therefore focused only on testing the GST–treacle fusion peptide containing exon 9.

We sought to determine whether the kinase activity that could phosphorylate the exon 9 GST–treacle fusion peptide was restricted to branchial arches I and II during early embryonic development. Crude tissue extracts from E3.5 limb buds, heart, stomach, extra-embryonic membrane, trunk (first somite to the tail bud, excluding visceral organs and limb buds) and head (tissue above first branchial arch, mainly brain and eye) were assayed for a kinase activity that could phosphorylate the exon 9 GST–treacle fusion peptide. Strong kinase activity was detected in limb bud, heart and stomach extracts, while a somewhat weaker activity was detected in extra-embryonic membrane and trunk extracts (Fig. 5). No kinase specific for the exon 9 GST–treacle fusion peptide was detected in the head tissue extracted from these embryos.

Having established a kinase activity in E3.5 tissue extracts, we investigated whether this kinase activity showed temporal restriction during embryogenesis. **In vitro** kinase assays indicated that at E5, the exon 9 GST–treacle fusion peptide, was phosphorylated by a kinase activity in the lower jaw tissue extracted from these embryos (Fig. 6). Phosphorylation in the E5 limb, heart and liver was present, but clearly at a lower level than at E3.5. No phosphorylation of the exon 9 fusion peptide was detected in the E5 stomach. The kinase activity continued to decline and by E8.5 was barely detectable in the lower jaw, heart, stomach and liver and no longer detectable in the limbs (Fig. 7).

**DISCUSSION**

Phosphorylation of treacle may be important for its function during craniofacial development (7,18,19,23). Although it was predicted that many of the repeat units within the **TCOF1**...
exons were potential CKII phosphorylation site motifs, there were also repeat units identified that did not contain typical phosphorylation sites for this enzyme (7). These repeats were suggested to be potential phosphorylation motifs for PKC or other kinases. For this study, three GST–treacle fusion peptides were constructed that represented possible kinase targets. Purification of the GST–treacle fusion peptides revealed that although both the exon 14 and exon 22 fusion peptides appeared as a single band, the GST–treacle fusion peptide containing exon 9 appeared as a double band. Since the fusion constructs had been sequenced and found to contain the correct exonic sequences, this double band representing the treacle peptide occurred in the extra-embryonic membrane and trunk section, whereas no phosphorylation of the exon 9 GST–treacle fusion peptide was observed in the head section extracted from these embryos.

In vitro kinase assays using commercially available kinases have demonstrated that treacle peptides can act as substrates for kinase activity, providing that the peptide contains a phosphorylation site motif for the particular kinase. In mice, peak expression of treacle is reported at E8.5–E9.0 in the regions of the developing branchial arches, but continues to be expressed later. The equivalent developmental stage in avian/chick embryos is E1.5–E2.5 (24). To determine whether avian branchial arches could be used as a source of kinase activity against treacle peptides, branchial arches I and II were isolated from slightly older (E3.5) quail embryos. In younger avian developmental stages, the branchial arches are morphologically indistinct and it is difficult to accurately dissect and isolate sufficient tissue. Using in vitro kinase assays we established that the GST–treacle fusion peptide containing exon 9 (but not exon 14 or exon 22) was a substrate for kinases in the branchial arch tissue. We have further demonstrated that this endogenous tissue kinase activity has target recognition similar to CKII (as judged by phosphorylation of the fusion peptides). In addition, this kinase activity is not restricted to the branchial arches but is present in many, but not all, tissues in the early embryo. Whereas the wide spatial distribution of the kinase activity that can phosphorylate treacle peptides continues, the level of activity declines as development proceeds. This suggests that the restricted range of tissues involved in TCS following mutation of a ubiquitously expressed gene are not the result of a branchial arch-restricted kinase activity. However, these results do indicate that the phosphorylation of treacle may be important for its function, especially during early embryogenesis.

Since the tissues affected in TCS patients are solely branchial arch derived, in addition to treacle being phosphorylated, the function of the protein during early embryogenesis may therefore be dependent on other cofactors found only in branchial arches I and II and not in any of the other tissues examined in this study. It is possible that by acting as a chaperone in protein transport, treacle may need to be phosphorylated to transport molecules from the cytoplasm to the nucleus and may rely on a cofactor that is branchial arch specific to return to the cytoplasm for the next round of transport. We must also consider that the in vitro kinase assays may not directly reflect the actual kinase activity in a biological model. In a biological model, the phosphorylating activity within the cells would most likely be controlled through inhibitory/activating factors located within defined intercellular compartments. Yet, in an in vitro system the cells of the
established that treacle is a molecule that can be phosphorylated and point towards investigating how phosphorylation is important for the function of treacle in craniofacial development.

**MATERIALS AND METHODS**

**PCR conditions for TCOFI exons**

The oligonucleotide primers used for PCR, each containing BamHI sites at their 5' and EcoRI sites at their 3' end to enable cloning, were as follows: exon 9, 5'-CCGGATCCGGAAGGCCCC and 3'-GGCCATTCTGCAGCTGCATT CATGGGCTGCCAG; exon 14, 5'-CCGGATCCCCCAGCACAG GGTGAAAACCTCAGTA and 3'-GGCCATTCTGGATTTGGAG GACCCCTGCTTGGCC; exon 22, 5'-CCGGATCCCTTCTCTC CCTCTCAGTTATATGACC and 3'-GGCCATTCTGGATT TAGGGGACAACATGCCTGC. PCR assays were performed using 1.25 U AmpliTaq DNA polymerase (Perkin Elmer, Melbourne, Australia). All PCRs were carried out in 50 µl reaction volume containing 50 ng of each primer, 50 ng genomic DNA (template), 0.2 mM dNTPs and PCR buffer (with 1.5 mM MgCl2). The samples were processed through 40 amplification cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s in a Gene Amp 2400 thermal cycler (Perkin Elmer). The final extension step was lengthened to 7 min.

**Construction of GST–treacle fusion peptides**

Each PCR product was directly ligated to TA cloning vector pCRII-1 (Invitrogen, Adelaide, Australia) and transformed into chemically competent INVαF′ Escherichia coli cells were carried out according to the manufacturer’s recommendations (Invitrogen). PCR fragments were subcloned into the GST gene fusion vector pGEX2T (Pharmacia Biotech, Sydney, Australia) following digestion with EcoRI and BamHI. The integrity of all GST fusion constructs was confirmed by sequencing.

**Expression and purification of the GST–treacle fusion peptides**

*Escherichia coli* strain JM109 containing the appropriate GST–treacle fusion expression plasmid was grown in Luria broth containing ampicillin (100 µg/ml final concentration) at 37°C to late log phase. GST fusion peptide expression was induced with the addition of isopropyl β-D-thiogalactoside (IPTG, 1 mM final concentration) and incubation at 37°C was continued for 3–4 h. The GST fusion peptides were purified via glutathione–Sepharose affinity chromatography using standard protocols (26). The glutathione–Sepharose 4B matrix (Pharmacia Biotech) was prepared according to the manufacturer for batch purification of GST proteins. Each fraction of eluted protein was stored in elution buffer (10 mM Tris–HCl, pH 8.0) in aliquots containing 10% glycerol. Prior to analysing the GST fusion products by SDS–PAGE, 2x SDS sample buffer [62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 50 mM dithiothreitol (DTT), 2.3% SDS, sometimes including protease inhibitor cocktail (Sigma, Sydney, Australia)] containing 19% bromophenol blue and 1.7% β-mercaptoethanol was added to each fusion peptide sample and the samples were denatured at 100°C for 5 min.
The fusion protein was visualized following staining with Coomassic brilliant blue.

**Avian tissue preparation**

Various tissues from E3.5, E5 and E8.5 quail (Coturnix coturnix japonica) embryos were dissected and stored in Ham’s F12 medium. Each tissue sample was incubated in 1 ml F12 containing 2 mg/ml Dispase II (Roche Molecular, Melbourne, Australia) and left on ice for 15 min. This was followed by a 10 min centrifugation at 4°C, 1000 r.p.m. in an Eppendorf centrifuge. The supernatant was removed and the pellet resuspended in 1 ml of 1 mM EDTA in Hank’s balanced salt solution. After a 15 min incubation on ice, the suspension was pelleted at 1000 r.p.m. for 10 min. After decanting the supernatant, the pellet was washed in 1 ml F12 medium, then centrifuged at 1000 r.p.m. for a further 10 min. This step was repeated twice. This procedure dissociated the cells for easier solubilization and removed extracellular matrix components from the complex preparation. After the final wash, cell counts for each tissue were taken so that the cell number per tissue sample could be equalized amongst the samples to be analysed. The pellet from the final wash was resuspended in the appropriate volume (based on calculations from the cell count) of NP-40 lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.3, 1% NP-40) containing 10 mM MgCl₂, 5 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was left on ice for 30 min with occasional vortexing prior to being used. In addition to the cell counts, a protein estimation for each tissue sample was carried out using the DC Protein Assay (Bio-Rad, Sydney, Australia), with the absorbance measured at 750 nm. The results obtained for the protein estimation were consistent with the cell counts obtained for each tissue analysed.

**In vitro phosphorylation using purified enzymes**

The CKII kinase assay was carried out in a 10 µl reaction, containing 100 µM CKII enzyme (Promega, Sydney, Australia), 1 µg substrate (either casein, histone H1, GST or fusion peptide), 50 mM HEPES buffer, pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 0.1% β-mercaptoethanol, 10 mM ATP, 100 mM MgCl₂ and 0.5 µl [γ³²P]ATP (10 mCi/ml). The PKC kinase assay was also carried out in a 10 µl reaction, containing 0.25 µM PKC enzyme (Roche Molecular), 20 mM Tris–HCl, pH 7.4, 0.1 mM PMSF, 0.1% β-mercaptoethanol, 10 mM ATP, 10 mM MgCl₂ and 0.5 µl [γ³²P]ATP (10 mCi/ml), with the addition of co-factors 20 µg/ml 1,2-dioleoyl-sn-glycerol (Sapphire Biosciences, Sydney, Australia), 500 µM CaCl₂ and 100 µg/ml phosphatidylserine. Both CKII and PKC kinase reactions were left to proceed for 30 min at 37°C and subsequently terminated with 1 µl of 0.5 M EDTA. All samples were stored in 2× SDS sample buffer (containing bromophenol blue and β-mercaptoethanol) and denatured at 100°C for 1 min prior to analysis by SDS–PAGE and autoradiography.

**In vitro phosphorylation using endogenous tissue kinases**

Kinase assays using tissues extracted from avian embryos of varying ages were carried out in a 20 µl reaction using 16 µl of tissue lysate, 2 µl of substrate (either GST control or fusion peptides) and 2 µl [γ³²P]ATP (10 mCi/ml). The reaction proceeded for 30 min at 37°C and was stopped by the addition of 1 µl of 0.5 M EDTA. The kinase reaction was transferred into an Eppendorf tube containing 100 µl of glutathione–Sepharose beads and the fusion peptide was semi-purified from the branchial arch lysate with two washes in phosphate-buffered saline (PBS). After removing the PBS from the final wash, the semi-purified samples were then resuspended in 15 µl of elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0), vortexed briefly and spun in a benchtop centrifuge at 2000 r.p.m. for 5 min. The supernatant (15 µl) containing the eluted fusion peptide was removed to a fresh tube and mixed with 15 µl of 2× SDS sample buffer (containing bromophenol blue and β-mercaptoethanol). All samples were stored at −20°C and denatured (100°C, 1 min) prior to analysis by SDS–PAGE.

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