Integrative and Comparative Biology, volume 50, number 4, pp. 528–535
doi:10.1093/icb/icq022

SYMPOSIUM

Evidence for the Local Evolution of Mechanisms Underlying Limb Regeneration in Salamanders

A. Acely Garza-Garcia,* Paul C. Driscoll* and Jeremy P. Brockes[1,†]

*Division of Molecular Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK; †Institute for Structural and Molecular Biology, Darwin Building, UCL, Gower Street, London WC1E 6BT, UK

From the symposium "Animal Regeneration: Integrating Development, Ecology, and Evolution" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2010, at Seattle, Washington.

[1]E-mail: j.brockes@ucl.ac.uk

Synopsis The most extensive regenerative ability in adult vertebrates is found in the salamanders. Although it is often suggested that regeneration is an ancestral property for vertebrates, our studies on the cell-surface three-finger-protein Prod 1 provide clear evidence for the importance of local evolution of limb regeneration in salamanders. Prod 1 is implicated in both patterning and growth in the regeneration of limbs. It interacts with well-conserved proteins such as the epidermal growth-factor receptor and the anterior gradient protein that are widely expressed in phylogeny. A detailed analysis of the structure and sequence of Prod 1 in relation to other vertebrate three-finger proteins in mammals and zebra fish supports the view that it is a salamander-specific protein. This is the first example of a taxon-specific protein that is clearly implicated in the mechanisms of regeneration. We propose the hypothesis that regeneration depends on the activity of taxon-specific components in orchestrating a cellular machinery that is extensively conserved between regenerating and non-regenerating taxa. This hypothesis has significant implications for our outlook on regeneration in vertebrates, as well as for the strategies employed in extending regenerative ability in mammals.

Introduction

It is difficult to understand why some animals are able to regenerate significant parts of their body whereas others are not (Sanchez Alvarado 2000; Brockes et al. 2001; Carlson 2007; Brockes and Kumar 2008; Bely and Nyberg 2009). This problem is somewhat unusual for present-day biology in that despite widespread interest, much of it directed toward the implications for regenerative medicine, there has been little or no substantial progress. The most extensive repertoire of regenerative ability among adult vertebrates is found in various species of salamander. This includes most notably the limb, but also the jaws (Ghosh et al. 1996), intestine (O'Steen 1958), ocular tissues (Grogg et al. 2006), and sections of the heart (Oberpriller and Oberpriller 1974). In the case of regeneration of limbs, about 50 species have been investigated and these fall into 3 of the 10 families of salamanders (Urodela) (Scadding 1977; Sessions and Larson 1987). Some claims have been made for the occurrence of non-regenerating species (Scadding 1977), but it seems likely that all salamanders do regenerate their limbs although the rate is much slower in some examples than in others (Young et al. 1983). Why is an adult salamander able to regenerate its limb and why is an adult mammal not able to do so?

The first possibility is that the regeneration of limbs evolved locally in salamanders, perhaps about the time when ancestral salamanders branched from the vertebrate tree in the Permian (Anderson et al. 2008). The second option is that regeneration by adults is properly regarded as an ancestral or primordial property of metazoa (Bely and Nyberg 2009), and while limb regeneration was selectively maintained in salamanders it was lost in other tetrapod vertebrates. It is interesting that the second possibility is significantly preferred to the first in discussions

Advanced Access publication April 21, 2010

of this issue. For example, the problem is often posed in terms of understanding why we have lost the ability to regenerate. One example of invertebrates that have been analyzed in this way is the Nadine annelids, a small group of aquatic polychaete species in which some examples are unable to regenerate anterior segments after transection (see contribution by Bely in this volume) (Bely and Wray 2001). For this group, the block occurs apparently at different stages in the events following transection, and in one case it can be circumvented by amputating through a segment that is undergoing the fission mode of asexual reproduction (Bely and Sikes 2009). The relevance of this model to the broader comparison of salamanders and mammals is not clear.

The most unequivocal evidence in favor of local evolution would be to identify molecules that are found only in regenerative species and are clearly implicated in the mechanism of regeneration. To date, the analysis of regeneration in different vertebrate and invertebrate contexts has stressed the role of molecules and pathways that are highly conserved and that are also present in non-regenerative species (Reddien et al. 2005; Sanchez Alvarado and Tsonis 2006; Brockes and Kumar 2008). It is possible that this aspect may have attracted selective attention because it readily connects regeneration to other aspects of metazoan biology. Here we describe the identification of a salamander-specific protein that appears to be important for certain aspects of limb regeneration. Its interactions with other proteins that are widely expressed in phylogeny suggests a hypothesis that underlines the importance of local evolutionary change, and brings regeneration into line with other examples of local evolution.

Identification and activities of Prod 1

Prod 1 was identified in a differential cDNA screen for sequences implicated in proximodistal identity in limb regeneration by newts (da Silva et al. 2002). Retinoic acid (RA) and precursor retinoids are able to convert distal blastemal cells to a more proximal identity over a narrow (approximately 2.5-fold) range of concentration (Maden 1982; Kim and Stocum 1986). The screen was initiated by identifying from a distal library about 300 different cDNAs that were significantly upregulated or down-regulated by RA. Since RA converts distal to proximal, an upregulated sequence should be expressed at higher levels in a proximal blastema (P > D), whereas a down-regulated sequence should be D > P. This proved to be a restrictive criterion that was fulfilled by six cDNAs only, three in the up category and three in the down (da Silva et al. 2002). Many studies of positional identity in salamanders have proceeded by confronting cells from different positions on an axis, for example, proximal and distal (Nardi and Stocum 1983). This work suggested that an important aspect of identity could be expressed at the cell surface and thereby detect differences between neighbors. After analysis of the six cDNAs from the screen, one apparently encoded a small protein linked to the cell surface by a GPI glycolipid anchor. The amino acid sequence of the protein identified it as a member of the three-finger protein (TFP) family by virtue of the characteristic spacing of its eight Cys residues, and the presence of key conserved hydrophobic residues and a C-terminal motif shared by the family. The TFP fold is a versatile scaffold for mediating protein–protein interactions and it is found in many secreted, transmembrane, and GPI-anchored proteins. The distinctive features of the domain allow a potentially complete inventory of TFPs to be made from bioinformatic interrogation of a genomic database. For example, there are predicted to be 45 members of the TFP superfamily in the human genome (Galat 2008).

In subsequent studies, Prod 1 has been shown to possess several activities that may be relevant to its roles during regeneration (Fig. 1).

PD engulfment

If a proximal blastema and a distal blastema are confronted in culture so as to form a conjugate, the proximal member will always engulf the distal, while two blastemas from the same level (both proximal, PP or both distal, DD) will adhere and form a stable boundary (Nardi and Stocum 1983). The response of the more proximal blastema in this assay was blocked by introducing antibody to Prod 1 into the culture medium, so that a PD conjugate behaves as if the two members are from the same level (da Silva et al. 2002). The response was also blocked by the enzyme PIPLC, which specifically releases proteins attached to GPI anchors, and this result is in agreement with the antibody experiments in implicating an anchored protein.

PD displacement

The distal cells of an axolotl blastema were labeled by focal electroporation, and shown to give rise to cells in the hand of the regenerate. If distal cells in the contralateral blastema are electroporated with a Prod 1 plasmid, they contribute to more proximal structures of the upper arm (Echeverri and Tanaka 2005).

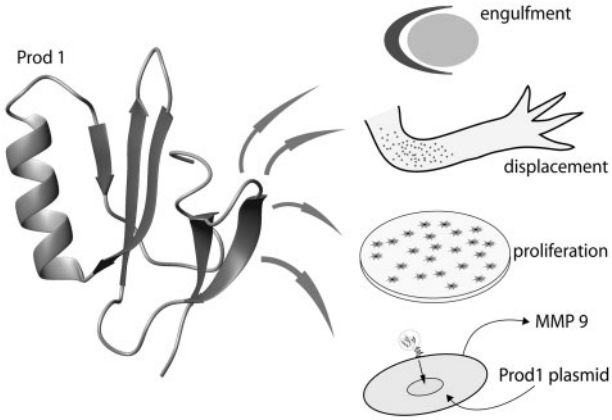

Fig. 1 Prod 1 and its activities. At left, a ribbon represents the solution 3D backbone structure of Prod 1; note the α -helix in Finger 3 (F3). The activities associated with Prod 1 are illustrated at the right hand side. Engulfment: a proximal limb blastema extends around a distal blastema that is in contact in culture (Nardi and Stocum 1983; da Silva et al. 2002). This is blocked by antibody to Prod 1 in the culture medium. Displacement: this refers to conversion of distal blastemal cells to proximal cells by expression of elevated levels of Prod 1 after electroporation. The cells take up a more proximal location than do control distal cells, which are essentially confined to the hand in the regenerated limb (Echeverri and Tanaka 2005). Proliferation: in the proliferation assay, cultured cells from the limb blastema are stimulated by recombinant nAG protein which acts through its surface receptor Prod 1 (Kumar et al. 2007). Signaling: the final example illustrates that expression of Prod 1 in cultured salamander cells activates a pathway leading to expression of MMP9.

Therefore, distal cells are converted to proximal by raising the level of expression of Prod 1.

Activation of a conserved signaling pathway

The GPI-anchored Prod 1 protein might be expected to interact with a transmembrane partner in order to signal to the interior of the cell. When Prod 1 was expressed by transfection of cultured limb cells of newts and axolotls, it activated a pathway leading to expression of the matrix metalloprotease 9 gene (MMP9) (R.Blassberg et al. submitted for publication). MMP9 is expressed during healing of wounds and regeneration, and MMP9 activity is necessary for regeneration to occur (Yang et al. 1999; Vinarsky et al. 2005). The pathway depends on activation of the epidermal growth factor receptor (EGFR), and subsequent MAP kinase signaling. Both newt and axolotl Prod 1 molecules have been shown to co-immunoprecipitate with newt EGFR when epitope-tagged versions of these molecules are expressed together. The EGFR is not the sole signaling partner but it can account for 30-40% of the activity required for MMP9 upregulation after Prod 1 expression.

Activity of the ligand nAG

Newt anterior gradient protein (nAG) was identified as a secreted ligand for Prod 1 in a yeast two-hybrid screen, and the recombinant proteins were subsequently shown to complex together (Kumar et al. 2007). nAG appears to play a key role in the nerve dependence of limb regeneration. The division of limb blastemal cells depends on the regeneration of axons in the major peripheral nerve branches following amputation (Singer 1952). These axons upregulate expression of nAG in Schwann cells of the distal nerve sheath, and subsequently, in dermal gland cells underlying the wound epithelium. The expression in both locations is abrogated if axonal regeneration is prevented by transecting the peripheral nerves at the base of the limb (Kumar et al. 2007). Importantly nAG has the critical ability to rescue regeneration of a denervated blastema so that it can complete the proximodistal axis and form digits. This molecule has provided a new focus for analyzing the mechanisms underlying the nerve dependence of limb regeneration, and how the relationship between the nerve and the limb is established during embryonic development.

The ability of the nAG protein to rescue the limb blastema probably reflects a direct action on the blastemal cells. Recombinant nAG protein stimulated cultured newt blastemal cells to enter S phase, and this activity was specifically inhibited by an antibody to Prod 1 (Kumar et al. 2007). Patterning and nerve-dependence are two aspects of limb regeneration that have been studied separately, although it is clear that the nerve is always required for regeneration to occur. The work with Prod 1 and nAG brings patterning and growth together at the molecular level as a protein–protein interaction.

Although we do not have a complete picture of the role of Prod 1 in regeneration, the experiments indicate that it acts in cell–cell interactions (displacement and engulfment), secreted ligand–cell interactions (nerve dependence), and signaling to the cell interior via interaction with the EGFR (Fig. 1).

Structure and phylogenetics of Prod 1

In order to understand more about its activities and its relationship to other members of the TFP family, the three-dimensional structure of Prod 1 has been determined by solution NMR (Garza-Garcia et al. 2009); a cartoon representation of the backbone fold is shown in Fig. 1. Prod 1 has the flat, disc-like shape that is characteristic of TFP domains. Its most distinctive feature is a 12-residue α-helix in the third finger. This is not unique among TFPs in that the

complement inhibitor CD59 and the C-terminal domain of mammalian urokinase plasminogen activator receptor (uPAR) also possess α -helices in the equivalent region (Garza-Garcia et al. 2009). Instead of the long single α -helix of Prod 1, these proteins present two small α -helices arranged almost perpendicularly to each other. There is evidence for the importance of this region of Prod 1 in signaling to the MMP9 pathway, as the mutation of two exposed residues in the α -helix of Prod 1 leads to the loss of >90% of the activity. Point mutations at other locations on the TFP disc have either no effect or a significantly lower impact (R. Blassberg et al., unpublished results).

Since the functional correspondence between equivalent proteins in different species is likely to be encoded in a similar 3D structure, an attempt was made to derive phylogenetic relationships based on the set of experimentally derived 3D structures of TFPs (Garza-Garcia et al. 2009). Such a tree indicates that the structures most similar to Prod 1 are CD59 and the C-terminal domain of uPAR. In addition to the α-helical region in the third finger described above, all three of these proteins are known to complex with the EGFR as analyzed by co-immunoprecipitation or proteomics approaches (Liu et al. 2002; Blagoev et al. 2003). Although Prod 1 was originally thought to be the newt ortholog of CD59 this assignment is clearly not correct, as the *Ambystoma* orthologs of CD59 have now been identified (Garza-Garcia et al. 2009). The manually curated structural superposition of these and other TFPs has been used to constrain the alignment of a much larger set of TFP domain amino-acid sequences, and to compute trees by both maximum likelihood and Bayesian analysis (Fig. 2A). This exercise has led to the conclusion that there is no ortholog of Prod 1 evident in mammals (Garza-Garcia et al. 2009).

These approaches have been further extended to analyze the TFP repertoire in the frog *Xenopus* as well as in the zebra fish, a model organism with considerable regenerative ability, for example, in the fins and heart (Poss et al. 2002; Tal et al. 2009). The analysis has been carried out on the sequences in the *Xenopus tropicalis* genome that are available to date (February 2010). No *Xenopus* ortholog to Prod 1 was evident (Fig. 2B). The results in the zebra fish case also show that it does not possess a TFP ortholog to Prod 1, but does have several groups of species-specific TFPs (Fig. 2C). Therefore, all available data suggest that Prod 1 is restricted to salamanders. It should be noted that orthologs to newt Prod 1 have been identified in *Ambystoma mexicanum* and *A. maculatum* (R. Blassberg et al., unpublished results), so that the arrival of Prod 1 in evolution must have been ancestral to the divergence of Salamandridae and Ambystomidae.

It could be argued that the species-specific contribution to the mechanism of regeneration is only involved in the fine tuning of the conserved, ancestral component. In contrast to this view, Prod 1 appears to be quite central to several aspects of regeneration that include patterning and nerve dependence. It seems likely that other examples of regeneration on the scale of the limb in different phyla will have a contribution from taxon-specific genes. One example is provided by the planarian *Schmidtea mediterranea* (Reddien et al. 2005) for which an RNAi screen of a sample of 1065 genes for phenotypes associated with tissue homeostasis and regeneration, yielded 240 genes that generated a relevant phenotype. Of these, 85% were predicted to encode proteins with significant homology to those encoded in other organisms, while 15% showed no homology and were regarded as taxon-specific. It is noteworthy in the context of the present discussion that it was the 85% that were the focus of attention for ''broadly informing general metazoan biology,'' including phenotypes arising from disease genes of humans (Reddien et al. 2005).

The TFP family provides several interesting examples of local evolutionary change that reflects expansion and diversification of taxon-specific proteins. The most intensively studied is that of the TFPs present in the venom of elapid snakes. These proteins display a wide variety of biological effects including inhibition of acetylcholine receptor (AChR) activity, cytotoxicity, and anticoagulation (Nirthanian and Gwee 2004; Kini 2006; Junqueira-de-Azevedo et al. 2006), but phylogenetic studies suggest that they evolved by gene duplication from a nontoxic ancestor probably able to bind nicotinic AChRs (Fry et al. 2003). The pharmacological properties of each of these groups of toxins depend on their binding specificity. For example, the type II α -neurotoxins cause paralysis by binding with high affinity and specificity to some muscular and neuronal AChR subtypes. An important determinant for AChR binding is an extended loop at the tip of the second finger (Fig. 3; Nirthanian and Gwee 2004). This aspect could be contrasted with the helical segment on Prod 1 which provides the basis for its signaling interaction with the EGFR and probably other signaling systems. The neurotoxin interaction inhibits the function of the AChR of the prey species, while the Prod 1 interaction is stimulatory for the

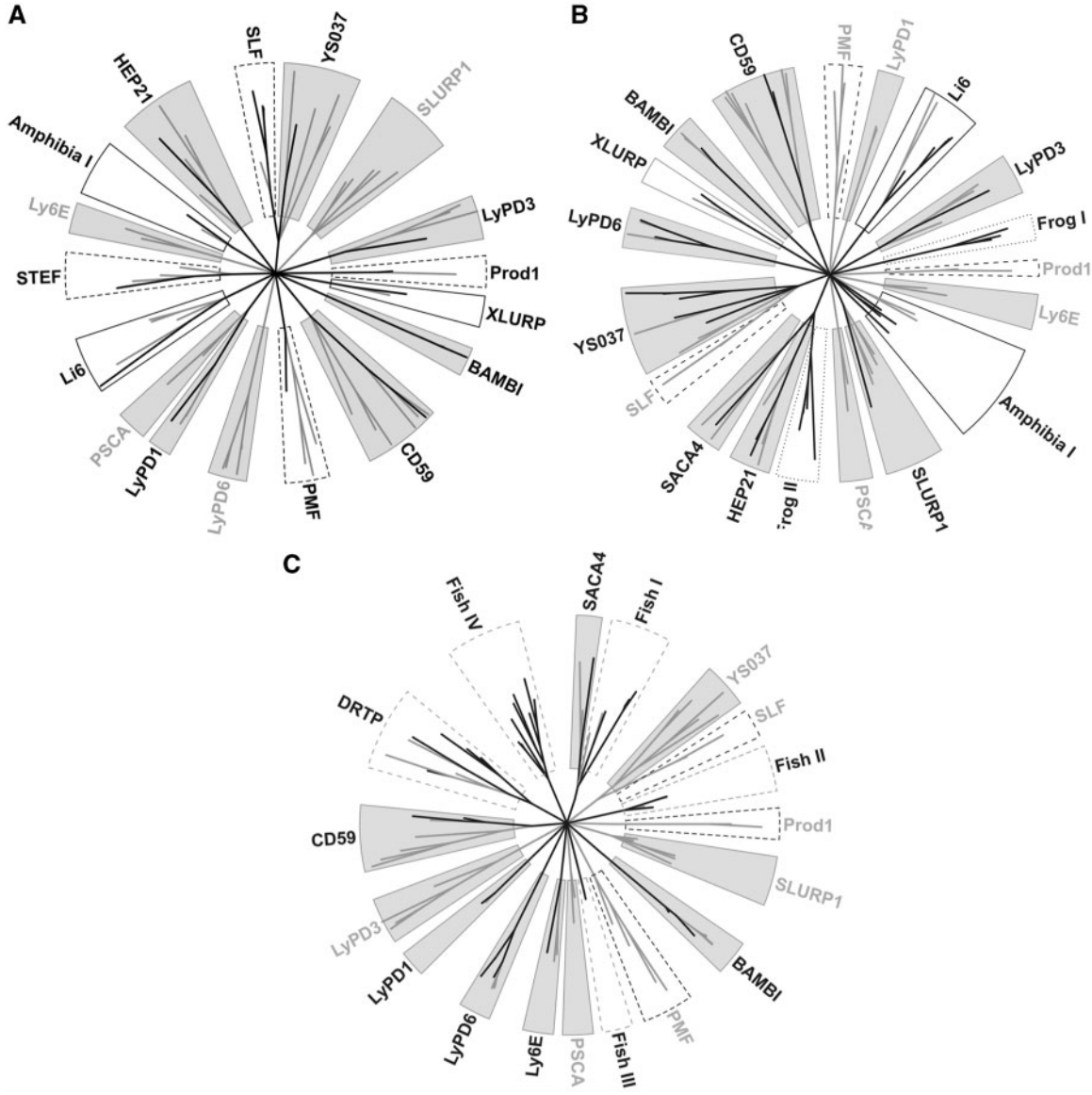

Fig. 2 Phylogenetic trees showing the occurrence of families of TFP domain-containing proteins in three vertebrates used as models for the study of regeneration. (**A**) The salamanders *Ambystoma mexicanum* and *tigrinum*, (**B**) the frog *Xenopus tropicalis* and (**C**) the zebra fish *(Danio rerio)*. Prod 1 is only identified in the salamanders. The model organism sequences were mined from the protein and EST databases at NCBI using blastp or blastn and a selection of representative members of the TFP superfamily, excluding families which were known to be specific to mammals. Kinase-containing proteins (activin receptor II family members) were removed due to the high computational cost of phylogenetic analyses. A multiple sequence alignment was computed with Muscle and adjusted manually, and the redundancy was reduced to >95% identity. The phylogenetic trees were calculated with Mr Bayes. In each tree, black branches represent proteins found in the model organism under consideration, and grey branches correspond to the sequences from other species used in construction of the tree. Each family of closely homologous proteins is contained in a bounded sector labeled with the name of a representative protein. If a protein belonging to a family is found in the model organism the sector label is black. A white background to the sector indicates that the family was found to occur in only one taxonomic order or taxonomic class, and (1) TFP families occurring only in salamanders have a black dashed border (STEF, salamander three finger proteins; SLF, sodefrin-like factors; PMF, plethodontid modulating factors). (2) TFP-families occurring only in frogs have a black dotted border, (3) only in amphibians, a solid black border, (4) only in fish, a dashed grey border. TFP-families with a grey background occur in more than one taxon. A table detailing the TFP proteins in the tree is given in the supplementary material. Prod 1 appears on the right of each tree at 3 o'clock, clearly delineated in a branch that lacks zebra fish or *Xenopus* sequences.

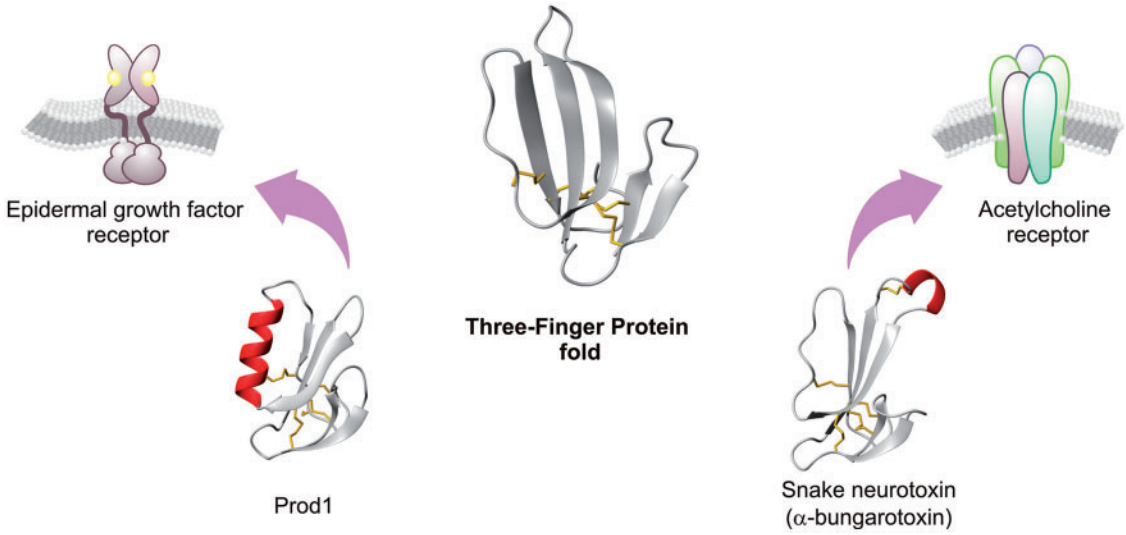

Fig. 3 Schematic representation of the diversification of the TFP fold in salamanders and elapid snakes. A typical TFP is shown in the middle with four disulfide bonds depicted in yellow. On the left the α -helical region in TFP Finger 3 is important in allowing Prod 1 to interact with EGFR signaling in salamander cells. On the right, the extended loop in TFP Finger 2 (red) provides a determinant that allows the α -neurotoxin family to interact with muscular and neuronal AChRs in various prey species (Nirthanian and Gwee 2004).

salamander EGFR. The similar association of CD59 and, particularly, of uPAR with EGFR presumably represent convergent evolution.

The principle of a taxon-specific and evolutionarily 'new' protein interacting with an 'old' conserved protein of widespread phylogenetic expression is a familiar and important one. In a study of the interactome data base for *Caenorhabditis elegans,* it was noted as a frequent occurrence, and suggested as a source of evolutionary innovation (Li et al. 2004). The evidence suggests that Prod 1 interacts not only with the EGFR but also with nAG, a thioredoxin superfamily member and a close homolog of a *Xenopus* protein involved in the specification of anterior ectoderm during embryonic development (Aberger 1998). The functional effect of introducing Prod 1 could be, in part, to tie together the activities of intracellular and intercellular signaling with a secreted ligand whose appearance during regeneration depends on the nerve supply.

This theme of interaction with a conserved cellular machinery is not limited to a direct physical association between protein molecules. In order for Prod 1 to play a role in PD identity during limb regeneration, it is critical that it be expressed in the right place at the right time. A remarkably conserved aspect of PD identity is the role of the Meis homeobox genes in regulating its expression. These have been implicated in this feature of limb development in *Drosophila*, chick and mouse, as well as in limb regeneration in the axolotl (Mercader et al. 1999, 2005). There are two consensus Meis binding sites in the axolotl Prod 1 promoter, and one of these has been shown by mutational analysis to play an important role in regulating the promoter in a proximal blastema (Shaikh et al., unpublished results). Therefore, the expression of the protein apparently depends on interaction with highly conserved *cis*-regulatory machinery.

Hypothesis about regeneration and phylogeny

The example of Prod 1 leads to a simplified hypothesis about the relationship between phylogeny and regenerative ability, presented here as three consecutive points.

(1) Regeneration depends on a largely conserved cellular machinery, familiar from processes such as development, wound healing, and tissue homeostasis. This machinery is present in both regenerative and non-regenerative taxa.

(2) This machinery is orchestrated by a relatively small number of taxon-specific components in ways that give a regenerative response to loss or injury of tissue.

(3) In salamanders, some or all of these components derive from expansion of the TFP family.

Point (1) represents a summary of much contemporary research on regeneration in different phylogenetic contexts, for example, hydra, planaria, amphibians, and fish. It raises the question of why regeneration on the scale of a limb can only occur in

a few taxa. Point (2) is perhaps the most critical part of the hypothesis. An alternative view would be that regeneration depends purely on *cis*-acting changes in regulatory sequences that alter the expression of the conserved machinery but do not depend on any taxon-specific components. Although research on regeneration has not emphasized taxon-specific genes and gene products, the examples from the planarian case described above, and the work on Prod 1, provide evidence that such will be discovered. A recent review has emphasized the widespread evolutionary importance of taxonomically restricted genes (Khalturin et al. 2009). Point (3) suggests that in the case of salamanders, order-specific members of the TFP family contribute to the orchestrating role described in Point (2), and Prod 1 is obviously a prototype in this regard. It would be very interesting if all such components in the salamander were revealed to be TFPs but this is perhaps unlikely. It seems probable that in other phylogenetic contexts this orchestrating role will be played by taxon-specific members of different gene families.

Recent studies have emphasized some of the distinctive features of the salamander genome as studied in *A. mexicanum* and *A. tigrinum* (Smith et al. 2009). On an average, a salamander gene is five times larger than a human gene, primarily because it has much longer introns. The frequency of paralogs in a selected axolotl–human dataset suggests that there are 2% more duplicated loci in the axolotl genome than in the human genome, and on an average more paralogs are predicted per duplicated locus. An example of expansion on a large scale is provided by the plethodontid modulating factors (PMFs), which are courtship pheromones expressed in the specialized mental gland in the chin of male lungless salamanders (Palmer et al. 2007). Over 100 unique haplotypes of this TFP family have been identified. Sequence comparisons suggest that the PMFs belong to a distinct multigene family within the salamander TFPs. Efforts are currently underway to analyze the remaining salamander-specific TFPs for their role in regeneration. It is possible that salamanders combine a genomic facility for managing these episodes of expansion and diversification, along with an ecology and life style that exerts strong selective pressure for regenerative ability, at least for regeneration of appendages.

The identification of a taxon-specific gene that plays an important role in limb regeneration in salamanders serves to question the assumptions behind our view of the process. If the salamander has brought significant evolutionary novelty to the problem of establishing a regenerative outcome to loss or injury of tissue, it seems inappropriate to ask why mammals have 'lost' the ability. Furthermore, if the present view is even partially correct, we should try to understand more about the taxon-specific input to the mechanism of regeneration, and the precise nature of orchestration. This would provide a more strategic view of the system-level problem of understanding limb regeneration. It is unlikely that this could be extended in any simple way to a mammalian context, but it would surely give an important impetus to such endeavors (Brockes and Kumar 2005).

Supplementary material

Supplementary material is available at *ICB* online.

Acknowledgments

The authors thank Robert Blassberg for help with Fig. 1. J.P.B. thanks Alexa Bely and Sara Lindsay for their invitation to speak at the Animal Regeneration Symposium.

Funding

Participation in the Animal Regeneration symposium was supported by NSF grant IOS-0940753 to Sara Lindsay and Alexa Bely; the Society for Integrative and Comparative Biology, including the divisions of Developmental and Cell Biology, Evolutionary Developmental Biology, Invertebrate Zoology, Ecology and Evolution; The American Microscopical Society; and the Society for Developmental Biology. Research in the PCD group at NIMR was supported by the MRC (file reference U117574559), and at UCL by an MRC Programme Grant and an MRC Research Professorship to J.P.B.

References

Aberger F, Weidinger G, Grunz H, Richter K. 1998. Anterior specification of embryonic ectoderm: the role of the Xenopus cement gland-specific gene XAG-2. Mech Dev 72:115–30.

Anderson JS, Reisz RR, Scott D, Frobisch NB, Sumida SS. 2008. A stem batrachian from the Early Permian of Texas and the origin of frogs and salamanders. Nature 453:515–18.

Bely AE, Nyberg KG. 2010. Evolution of animal regeneration: re-emergence of a field. Trends Ecol Evol 25:161–70.

Bely AE, Sikes JM. 2010. Latent regeneration abilities persist following recent evolutionary loss in asexual annelids. Proc Natl Acad Sci USA 107:1464–9.

Bely AE, Wray GA. 2001. Evolution of regeneration and fission in annelids: insights from engrailed- and orthodenticle-class gene expression. Development 128:2781–91.

Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M. 2003. A proteomics strategy to elucidate functional protein–protein interactions applied to EGF signaling. Nat Biotechnol 21:315–18.

Brockes JP, Kumar A. 2005. Appendage regeneration in adult vertebrates and implications for regenerative medicine. Science 310:1919–23.

Brockes JP, Kumar A. 2008. Comparative aspects of animal regeneration. Annu Rev Cell Dev Biol 24:525–49.

Brockes JP, Kumar A, Velloso CP. 2001. Regeneration as an evolutionary variable. J Anat 199:3–11.

Carlson BM. 2007. Principles of regenerative biology. Amsterdam: Academic Press.

da Silva SM, Gates PB, Brockes JP. 2002. The newt ortholog of CD59 is implicated in proximodistal identity during amphibian limb regeneration. Dev Cell 3:547–55.

Echeverri K, Tanaka EM. 2005. Proximodistal patterning during limb regeneration. Dev Biol 279:391–401.

Fry BG, Wuster W, Kini RM, Brusic V, Khan A, Venkataraman D, Rooney AP. 2003. Molecular evolution and phylogeny of elapid snake venom three-finger toxins. J Mol Evol 57:110–29.

Galat A. 2008. The three-fingered protein domain of the human genome. Cell Mol Life Sci 65:3481–93.

Garza-Garcia A, Harris R, Esposito D, Gates PB, Driscoll PC. 2009. Solution structure and phylogenetics of Prod1, a member of the three-finger protein superfamily implicated in salamander limb regeneration. PLoS One 4:e7123.

Ghosh S, Thorogood P, Ferretti P. 1996. Regeneration of lower and upper jaws in urodeles is differentially affected by retinoic acid. Int J Dev Biol 40:1161–70.

Grogg MW, Call MK, Tsonis PA. 2006. Signaling during lens regeneration. Semin Cell Dev Biol 17:753–8.

Junqueira-de-Azevedo IL, Ching AT, Carvalho E, Faria F, Nishiyama MY Jr, Ho PL, Diniz MR. 2006. Lachesis muta (Viperidae) cDNAs reveal diverging pit viper molecules and scaffolds typical of cobra (Elapidae) venoms: implications for snake toxin repertoire evolution. Genetics 173:877–89.

Khalturin KG, Hemmrich S, Fraune R, Augustin R, Bosch T. 2009. More than just orphans: Are taxonomically-restricted genes important in evolution? Trends Genet 25:404–13.

Kim WS, Stocum DL. 1986. Retinoic acid modifies positional memory in the anteroposterior axis of regenerating axolotl limbs. Dev Biol 114:170–9.

Kini RM. 2006. Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem J 397:377–87.

Kumar A, Godwin JW, Gates PB, Garza-Garcia AA, Brockes JP. 2007. Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. Science 318:772–7.

Li S, et al. 2004. A map of the interactome network of the metazoan C. elegans. Science 303:540–3.

Liu D, Aguirre Ghiso J, Estrada Y, Ossowski L. 2002. EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. Cancer Cell 1:445–57.

Maden M. 1982. Vitamin A and pattern formation in the regenerating limb. Nature 295:672–5.

Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, Torres M. 1999. Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature 402:425–9.

Mercader N, Tanaka EM, Torres M. 2005. Proximodistal identity during vertebrate limb regeneration is regulated by Meis homeodomain proteins. Development 132:4131–42.

Nardi J, Stocum DL. 1983. Surface properties of regenerating limb cells: evidence for gradation along the proximodistal axis. Differentiation 25:27–31.

Nirthanian S, Gwee M. 2004. Three-finger alpha neurotoxins and the nicotinic acetylcholine receptor, forty years on. J Pharmacol Sci 94:1–17.

O'Steen W. 1958. Regeneration of the intestine in adult urodeles. J Morphol 103:435–78.

Oberpriller JO, Oberpriller JC. 1974. Response of the adult newt ventricle to injury. J Exp Zool 187:249–53.

Palmer C, Hollis D, Watts R, Houck L, McCall M, Gregg R, Feldhoff P, Feldhoff R, Arnold S. 2007. Plethodontid modulating factor, a hypervariable salamander courtship pheromone in the three-finger protein superfamily. FEBS J 274:2300–10.

Poss KD, Wilson LG, Keating MT. 2002. Heart regeneration in zebrafish. Science 298:2188–90.

Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sanchez Alvarado A. 2005. Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell 8:635–49.

Sanchez Alvarado A. 2000. Regeneration in the metazoans: why does it happen? Bioessays 22:578–90.

Sanchez Alvarado A, Tsonis PA. 2006. Bridging the regeneration gap: genetic insights from diverse animal models. Nat Rev Genet 7:873–84.

Scadding S. 1977. Phylogenetic distribution of limb regeneration capacity in adult Amphibia. J Exp Zool 202:57–68.

Sessions S, Larson A. 1987. Developmental correlates of genome size in Plethodontid salamanders and their implications for genome evolution. Evolution 4:1239–51.

Singer M. 1952. The influence of the nerve in regeneration of the amphibian extremity. Q Rev Biol 27:169–200.

Smith JJ, Putta S, Zhu W, Pao GM, Verma IM, Hunter T, Bryant SV, Gardiner DM, Harkins TT, Voss SR. 2009. Genic regions of a large salamander genome contain long introns and novel genes. BMC Genomics 10:19.

Tal TL, Franzosa JA, Tanguay RL. 2010. Molecular signaling networks that choreograph epimorphic fin regeneration in Zebrafish—a mini-review. Gerontology 56:231–40.

Vinarsky V, Atkinson DL, Stevenson TJ, Keating MT, Odelberg SJ. 2005. Normal newt limb regeneration requires matrix metalloproteinase function. Dev Biol 279:86–98.

Yang EV, Gardiner DM, Carlson MR, Nugas CA, Bryant SV. 1999. Expression of Mmp-9 and related matrix metalloproteinase genes during axolotl limb regeneration. Dev Dyn 216:2–9.

Young HE, Bailey CF, Dalley BK. 1983. Gross morphological analysis of limb regeneration in postmetamorphic adult Ambystoma. Anat Rec 206:295–306.