Commentary

Hormone and growth factor subunits: a novel perception of cell growth regulation

R. T. Radulescu and C. M. Wendtner

The purpose of this communication is to present some novel insights into putative mechanisms underlying autocrine cell growth control. These concepts are founded on sequence homologies between cellular growth factors and viral oncoproteins as determined by previous studies (Waterfield et al. 1983) and our recent work (Radulescu & Wendtner, 1992), as well as experimental data demonstrating the profound impact of hormones/growth factors and their fragments on cell differentiation and proliferation (Sporn & Roberts, 1985; Rosen, 1987; Cross & Dexter, 1991).

Proposed interaction between insulin and retinoblastoma protein

An important factor was our recent discovery of the sequence leucine-X-cysteine-X-glutamic acid (LXCXE; X=any amino acid) in the B-chain of the pancreatic hormone insulin (Radulescu & Wendtner, 1992). This sequence is identical with the motif proven to be essential for binding of viral oncoproteins to retinoblastoma protein (RB) (Jones et al. 1992), a tumour suppressor gene product shown to be crucially involved both in embryogenesis (Lee et al. 1992) and oncogenesis of many different tumours apart from retinoblastoma (Weinberg, 1991; Harlow, 1992). Moreover, we have detected a hydrophilically related motif in the insulin-related growth factors, insulin-like growth factor I and II (IGF-I and -II), but not in nerve growth factor and relaxin that are also members of the insulin family. We have further predicted that insulin, IGF-I and IGF-II may bind to sites on RB and p107, an RB-related protein (Ewen et al. 1991), identical to those previously proposed (Wendtner & Radulescu, 1993) for viral oncoproteins and cellular ligands. This assumption is based on the hydrophilic complementarity approach (Bost et al. 1985) that arose from the observation that codons for hydrophobic amino acids on one strand of DNA are complemented by codons for hydrophilic amino acids on the other strand, and vice versa (Blalock & Smith, 1984). Hence, peptides derived from the same reading frame of complementary strands of nucleic acids possess an inverted hydropathy which may induce amphiphilic structures and promote binding (Fassina et al. 1989). The specific binding of peptides hydrophilically complementary to each other has been validated extensively (Kang et al. 1988; Blalock, 1990; Tropsha et al. 1992).

Interestingly, the analogy between the LXCXE sequences in insulin and in other proteins (Figge et al. 1993) holds at the level of the secondary structure too, since the LXCXE sequences both in the insulin B-chain (Perutz, 1992) and in various RB-binding proteins, including viral oncoproteins, have an element of α-helix. However, it should be emphasized that, to date, it is not known whether the active structure of an RB-binding peptide is induced upon interaction with RB, is a precondition, or is a combination of both (Figge et al. 1993). The environment may play a pivotal role in inducing the conformation of RB-binding peptides, as has already been demonstrated with other peptides (Zhong & Johnson, 1992).

Based on our identification of the LXCXE sequence in insulin and of related motifs in IGF-I and IGF-II, we concluded that these molecules may stimulate growth through complexing and inactivating RB and/or p107. Similar to the action of viral oncoproteins like the E7 protein of human papilloma virus type 16 (HPV-16 E7), the predicted complex formation between insulin/IGF-I/IGF-II and RB respectively, might result in the displacement of E2F, a cellular factor with transcriptional activating capacity, from its RB-binding site (Chellappan et al. 1992). This would enable the liberated transcription factor to interact with DNA promoter regions and subsequently to switch on gene expression (Weintraub et al. 1992). In other words, insulin would inhibit the transcriptional repressor activity of RB by liberating transcription factors with positive growth regulatory action from the RB ‘trap’. More generally speaking, our prediction of associations
between insulin/IGF-I/IGF-II and RB/p107, respectively, could represent a possible mechanism underlying the well-known involvement of these factors in cell cycle progression (Pardee, 1989; Cobrinik et al. 1992). Thus, insulin, IGF-I and IGF-II, all of which contain the LXCXE sequence or a hydrophathically similar variation, would resemble the viral oncoproteins E1A of adenovirus type 5 (Ad5 E1A) and large T antigen of simian virus type 40 (SV40 large T) that use the same sequence to target two points of E2F regulation, i.e. RB and p107 (Cao et al. 1992).

There are at least four conceivable pathways through which insulin may interfere with the physiological functions of RB. First, especially in the case of an overexpression of the insulin gene, some insulin molecules may stay intracellular and directly associate with RB as suggested by us (Radulescu & Wendtner, 1992). Our proposed complex formation between insulin and RB may thus occur without prior secretion of insulin. We therefore infer that de-novo synthesized insulin may act as an autocrine growth factor or even as a transcription factor in many different cells, normal and neoplastic, by staying intracellular and assembling with RB intracellularly. This novel concept, if validated, would provide an important extension of the growth-regulatory pathways presently assigned to insulin. To date, insulin is still thought in terms of a pancreatic hormone that systemically promotes growth of target tissues through facilitating cellular glucose utilization as well as through generating second messengers and switching on post-receptor phosphorylation events (Rosen, 1987). Secondly, insulin may enter the cell through a cell membrane receptor-independent mechanism as recently demonstrated by Lin et al. (1992). These authors suggested that the internalized insulin might interact with intracellular ligands with transcriptional control activity. Yet a third possibility is the intracellular assembly between the internalized insulin–insulin receptor complex and RB. This assumption is supported by a study demonstrating nuclear translocation of the insulin receptor (Podlecki et al. 1987). Through electron microscopic radioautography, evidence was provided for the accumulation of the insulin–insulin receptor complex at the periphery of the condensed chromatin of the nucleus, parallel studies showing a similar localization of progesterone receptors and of internalized insulin (Podlecki et al. 1987). The proposal of a trimolecular complex consisting of insulin, insulin receptor and RB is also consistent with our data because analysis of the insulin B-chain revealed that the domain proposed to bind to RB and the region known to interact with the insulin receptor are not identical or overlapping, but contiguous (Radulescu & Wendtner, 1992). In this complex, the LXCXE sequence in the B-chain of insulin would recruit RB in the vicinity of the internalized insulin receptor so that the latter could phosphorylate RB through its intrinsic tyrosine kinase activity. This proposed event would parallel the recruitment of intracellular signal transduction molecules by complexes consisting of cell membrane-bound/internalized (Cross & Dexter, 1991) growth factor receptors and of cellular proteins carrying certain recognition domains, referred to as src homology-2 (SH2) domains (Brugge, 1993). Another scenario preceding a putative interaction between insulin and RB would be that insulin enters the cell by means of the internalized insulin–insulin receptor complex and then dissociates from it.

RB may not only be inactivated through a physical association with insulin and/or the insulin–insulin receptor complex, but also by insulin-induced phosphorylation through the tyrosine kinase activity of the cell membrane-bound insulin receptor, since RB is known to be functionally silenced by cell cycle-dependent hyperphosphorylation (Cobrinik et al. 1992). This alternative mechanism may involve signal transduction through serine-threonine kinases, since this group of molecules is both a phosphorylation target for cell surface-associated receptors with tyrosine kinase activity (Schindler et al. 1992) and an enzyme family (cyclic-dependent kinases) capable of phosphorylating RB in a cell cycle-dependent manner (Cobrinik et al. 1992). This mode of action would provide a direct link from a cell membrane receptor to the nucleus similar to the interferon-α-mediated phosphorylation and activation of a cytoplasmic transamination factor (Schindler et al. 1992).

The validity of our prediction regarding a potential interaction between insulin and RB remains to be determined. Future studies will have to elucidate whether human preproinsulin, proinsulin, the insulin B-chain alone and the active insulin molecule all interact with RB.

From the insulin B-chain to the subunit hypothesis

Landmark gene cloning work done in several laboratories over the past decade has revealed unexpected sequence similarities between growth factors and their receptors on the one hand and viral oncoproteins on the other (Sporn & Roberts, 1985). Two examples are the homology between the B-chain of platelet-derived growth factor (PDGF) and the v-sis oncprotein of simian sarcoma virus (VSGF), as well as the homology between the thyroid hormone/oestrogen receptor and the v-erbA oncprotein of avian erythroblastosis virus on the receptor side (Evans, 1988).

In the light of these well-established data, we now suggest that the hormone insulin, its precursors
and/or the insulin B-chain alone may be perceived as products of a cellular proto-oncogene. Their viral counterparts could be HPV-16 E7, Ad5 E1A, SV40 large T and/or other heretofore unidentified viral oncoproteins. Based on these assumptions, it may be rewarding to screen tumours for overexpression of insulin, insulin precursors and the insulin B-chain and to correlate the respective findings with the degree of malignancy. Interestingly, a substance immunologically cross-reactive with insulin (SICRI) has already been purified from human and murine in-vivo tumours as well as from murine myeloid leukaemia and melanoma cell lines (Bajzer et al. 1984; Levanat & Pavelics, 1990). The authors (Bajzer et al. 1984) suggested that SICRI may stimulate cell growth in two ways: through the intermediary of growth hormone released into the circulation following SICRI-induced systemic hypoglycaemia and by embarking on an autocrine pathway.

Screening for overexpression of the insulin B-chain in tumours appears to be warranted in the context of previously demonstrated up-regulations of specific hormone/growth factor fragments and their receptors in neoplastic tissues. Examples of this phenomenon are the selective coexpression of PDGF B-chain and PDGF B-type receptor in various human gliomas (Mauro et al. 1991) as well as the selective expression of PDGF B-type receptors in mesotheliomas (Versnel et al. 1991). Moreover, it remains to be determined whether the independent overexpression of the α- and β-subunits of follicle-stimulating hormone, luteinizing hormone and thyroid-stimulating hormone in pituitary tumours (Black et al. 1987) is an incidental phenomenon or a reflection of their intracellular/extracellular involvement in growth control. A rather ubiquitous distribution of hormone fragments in many different tumours has already been demonstrated for opioid peptides which are cleaved from a precursor protein termed pro-opiomelanocortin (POMC) (Bostwick et al. 1987). These authors pointed out that these peptide subunits are useful markers of neuroendocrine differentiation and possibly have autocrine roles, for example, in small-cell lung carcinoma.

We therefore propose that hormone/growth factor subunits, perhaps more so than the complete hormones, are crucially involved in cellular transformation. This subunit hypothesis provides an interesting parallel to the well-established role of inositol phosphates as second messengers. Similar to these molecules which are functional breakdown products of cell membrane phospholipids (Berridge & Irvine, 1989), hormone and growth factor subunits may be regarded as biologically active fragments of their precursors. This analogy particularly holds if one assumes that these fragments have transient and yet metabolically relevant effects. The proposed signal transduction pathway through hormone subunits has previously remained undetected (Cross & Dexter, 1991) and will therefore require more sensitive detection systems.

The experimental validation of intracellular growth-regulatory properties for the insulin B-chain would be an important addition to our concept according to which hormone and growth factor fragments may influence cell metabolism. Consistent with our hypothesis, Lin et al. (1992) did not rule out intracellular influences of partially processed insulin fragments on gene transcription. In contrast, Miller's data (1989) were suggestive of a functional role for intracellular insulin but not for the insulin B-chain in the stimulation of protein synthesis. An appropriate way to test the intracellular functions of the insulin B-chain may be to transfect the gene fragment coding for it, rather than to microinject the B-chain protein (Miller, 1989), into cells and measure changes in proliferation subsequent to its expression. In fact, Miller's data (1989) do not exclude that, in the reducing environment of the intracellular space, insulin breaks down into A- and B-chain and the B-chain is the component to mediate the observed insulin actions physically. Conversely, the microinjection of the insulin B-chain (Miller, 1989) might have been without effect because of a conformational instability induced by the method itself or because of a more rapid degradation of the B-chain versus the insulin molecule following injection. An alternative approach to test the functionality of the B-chain inside the cell would be to design an experiment in which the effects of blocking, by means of synthetic antisense oligonucleotide sequences, the messenger RNAs coding for the A-chain and the entire insulin molecule would be compared with each other.

Intracrine cell growth regulation

The above data lead us to hypothesize that, under the conditions of accelerated and increased protein synthesis associated with neoplastic transformation, only a part of the produced proteins is secreted by the cell and proceeds to interact with cell membrane receptors of the same cell (classical autocrine cell growth hypothesis) (Sporn & Roberts, 1985). A certain proportion of the overexpressed proteins, however, may not engage in secretory processes since saturation of carrier molecules, for example, of ATP-binding-cassette (ABC) transport proteins (Kuchler & Thorner, 1992), may occur rapidly. Hence, these proteins would stay intracellular and subsequently associate with other cytoplasmic or nuclear ligands. Applying this concept to hormone/growth factor subunits implies that not all fragments would be

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assembled to a hormone complex consisting of several different subunits. Some fragments may associate with other intracellular proteins possessing appropriate binding sites and thereby switch on an internal autoactivation mechanism. One example for this scenario is the proposed interaction between insulin B-chain and RB (Radulescu & Wendtner, 1992). Alternatively, identical subunits may bind one another, thus forming homodimers. For instance, it is conceivable that insulin B-chain homodimers may be formed, similar to the PDGF B-chain homodimers (Antoniades, 1991; Bejcek et al. 1992).

There might be more specific ways of keeping a newly synthesized peptide intracellular. This could be achieved, for example, by a short carboxy-terminal extension of the actual growth factor leading to its retention within the endoplasmic reticulum, as demonstrated with modified interleukin-3 (IL-3) (Dunbar et al. 1989). The introduction of the gene coding for the modified IL-3 by retroviral gene transfer into IL-3-dependent haematopoietic cells made these cells become growth factor-independent, despite lack of secretion of the modified IL-3. The recent demonstration of an intracellular co-localization of active PDGF precursors and PDGF receptors has prompted investigators to postulate the existence of an internal autocrine pathway independent of PDGF secretion (Silver, 1992). It appears to be true, at least for PDGF, that its strong transforming stimulus is conveyed through intracellular pathways rather than through interactions with cell membrane receptors since even large amounts of exogenous PDGF cannot reproduce the potent proliferative signal derived from its proto-oncogene counterpart c-sis (Bejcek et al. 1989).

A study showing rapid nuclear localization of fibroblast growth factor (FGF) associated with an increase in gene transcription (Bouche et al. 1987) also supports the assumption of an intracellular autocrine pathway. This finding was extended by showing that the int-2 gene which is the proto-oncogene coding for an FGF-like protein, generates two alternative translation products, one of which is targeted to the nucleus (Acland et al. 1990). The form of FGF that contains CUG as an initiation codon for its mRNA comprises a sequence that mediates translocation to the cell nucleus (nuclear targeting sequence) and has a higher molecular weight than the cytoplasmic variant of FGF which is encoded by an AUG-initiated mRNA (Amalric et al. 1991). The latter form of FGF, an 18 kDa protein, has recently been demonstrated to be released from living cells and to affect FGF-dependent cell migration through the interaction with cell membrane receptors (Rifkin et al. 1991). These receptors have associated tyrosine kinase activity (Cross & Dexter, 1991) through which, in addition to intracellular autocrine pathways, FGF can stimulate growth (Neufeld & Gospodarowicz, 1985). This finding has been extended by experimental data suggesting that extracellular FGF bound to its cell membrane receptor may be activated through phosphorylation by an ecto-protein kinase associated with the surface of a human hepatoma cell line (Vilgrain & Baird, 1991).

The lack of a signal peptide sequence (normally required for the transport of the respective protein into the endoplasmic reticulum and its subsequent secretion into the extracellular environment) in some growth factors, has prompted investigators (Logan, 1990) to suggest that this might commit these molecules to an intracellular mechanism of action. Since, however, alternative pathways of secretion have been demonstrated for a number of proteins lacking a signal sequence (Kuchler & Thorner, 1992), we propose that these peptides are bifunctional: they may act as classical autocrine/paracrine growth factors through secretion followed by interaction with cell membrane receptors and/or they may be involved in intracellular cell growth regulation.

The above data lead us to conclude that there might be a ‘fast track’ or ‘short cut’ to the genes, in that their expression may be influenced by intracellular complexes formed between hormone/growth factor subunits and their respective ligands. Under conditions of tumorigenesis this may be an important cause of uncontrolled cell growth, probably equal in significance to the other two already postulated causes: secreted growth factors stimulating the growth of the secreting cell (classical autocrine loop) (Sporn & Roberts, 1985) and the constitutive growth impetus conferred by truncated hormone/growth factor receptors encoded by viral oncogenes (for example, the truncated epidermal growth factor receptor encoded by v-erbB) (Ullrich et al. 1984). The shortening of the autocrine pathway potentially resulting from hormone subunits interacting with ligands intracellularly, thus bypassing secretion and the subsequent interaction with cell membrane receptors, may represent a sequential step further in the acceleration of growth ensuing from replacing paracrine mechanisms by autocrine impulses. A switch from paracrine to autocrine growth mechanisms occurs in the following setting. Normal adrenal cortical cells and myoblasts, for instance, require exogenous FGF for growth (Gospodarowicz et al. 1987). However, mouse Y-1 adrenal cortical tumour cells and A204 rhabdomyosarcoma cells can proliferate without exogenous basic FGF because of their ability to produce and to respond to their own FGF (Schweigerer et al. 1987). Yet another example is that, in the normal thyroid, stromal cells produce IGF-I which acts on follicular cells in a paracrine manner. In contrast, the neoplastic
cells of thyroid adenomas are not dependent on neighbouring cells as a source of IGF-I. Instead, they bypass the need for this growth factor by producing IGF-I themselves in an autocrine fashion (Williams, 1988). IGF-I may also mediate a switch from autocrine to intracellular autocrine growth mechanisms. Experimental data show that monoclonal antibodies blocking the IGF-I receptor could inhibit anchorage-independent growth in most cell lines only in the presence of serum, but not under serum-free conditions (Arteaga, 1992).

Interestingly, it has been proposed that this intracellular autocrine pathway exists for steroid hormone receptors and their ligands (O'Malley, 1989; O'Malley & Conneely, 1992) and has accordingly been coined 'intracrine pathway' (O'Malley, 1989). This is supported by our identification (R. T. Radulescu & C. M. Wendtner, unpublished observation) of sequences hydropathically related to the LXCXE RB-binding motif in the first zinc finger of the DNA binding domains of members of the steroid and thyroid receptor superfamily (Evans, 1988), suggesting that these transcription factors might influence gene expression through an (intracellular) association with RB. Moreover, our assumption may provide a molecular basis for the postulated oncogenic potential of steroid hormone receptors (Green & Chambon, 1986). Consistent with our hypothesis are also experimental data demonstrating that steroid receptors interact with many transcription factors in order to achieve efficient transcriptional activity (Schüle et al. 1988; Shemshedin et al. 1992). In accordance with our proposed binding domain for RB on steroid receptors, an investigation into the modulation of glucocorticoid receptor function by protein kinase A (PKA) (Rangarajan et al. 1992) suggested that the site of PKA interaction was the DNA-binding domain. Conversely, RB has been shown to interact functionally with Sp1, a zinc finger transcription factor (Udvadia et al. 1993), thereby providing an additional analogy to our proposed interaction between RB and the zinc finger proteins of the steroid/thyroid hormone receptor superfamily.

The proposed interaction between members of the steroid/thyroid receptor superfamily and RB may represent an additional mechanism, other than a phosphorylation cascade, accounting for the demonstrated ligand-independent activation of steroid/thyroid receptors (Power et al. 1991a,b). The dopamine-induced nuclear translocation and phosphorylation of steroid hormone receptors might also coincide with a conformational change of these molecules conducive for interaction with RB. Similar to the action of viral oncoproteins, the proposed binding of these receptors to RB would displace E2F from its RB-binding site and therefore promote cell proliferation. Conversely, the interaction between steroid receptors and their natural ligands may induce receptor conformations preventing binding to RB and thus maintain a transcriptionally inactive RB–E2F complex with the net result of cell differentiation. This would be consistent, for example, with a study reporting that the oestrogen–oestrogen receptor complex mediates differentiation of a neuroblastoma cell line (Ma et al. 1993).

The LXCXE hydrophatic superfamly

Based on our discovery of the LXCXE motif in insulin and its hydropathically similar variations in IGFs as well as in members of the steroid/thyroid receptor superfamily, we hypothesize that this consensus sequence may be found in several other proteins involved in intracellular growth regulation. This would add to the structure–function relationship – already substantiated by the leucine zipper and the SPXX motif (Perutz, 1992) – in processes underlying cell differentiation and proliferation.

Moreover, we have noted another intriguing similarity between the LXCXE sequence in insulin and the motif variant in members of the steroid/thyroid receptor superfamily. In all these molecules, the cysteine residue present in the LXCXE motif and the LXCXE-related sequences respectively, is structurally constrained and forms the bottom of a groove with the amino- and carboxy-terminal residues of these sequences being exposed to the solvent (Blundell et al. 1971; Luisi et al. 1991). In insulin, the respective cysteine participates in the disulphide bond between the A- and B-chain, whereas in steroid and thyroid receptors it is involved in binding to a zinc ion, thus contributing to the formation of the first zinc finger of the DNA-binding domain of these receptors. This implies that the cysteine both in the LXCXE motif in insulin and the LXCXE-related motif in steroid/thyroid receptors is not likely to be free to form potential disulphide bridges to RB. This assumption is in accordance with experimental evidence provided by Jones et al. (1990) who have demonstrated that the cysteine contained in the LXCXE motif of the HPV-16 E7 viral oncoprotein, although essential for binding to RB, does not interact with RB through a disulphide linkage. It rather seems to participate in complex formation with RB through the peptide bond amino group since methylation of this amino group resulted in a profound loss of binding potency (Jones et al. 1992).

Previous studies have identified cellular proteins that bind to RB (Kaelin et al. 1991) prior to the detection of the LXCXE sequence in these proteins through structural analysis (Deleo-Jones et al. 1991; Dowdy et al. 1993). A reverse approach has been
employed by Leiden and colleagues (Kaelin et al., 1992; Wang et al., 1993) who initially detected the LXCXE motif in the cellular transcription factor Elf-1 and then demonstrated that Elf-1 can bind specifically to RB in vitro, provided that the RB-binding domain is intact. Hence, the predictive value of the presence of the LXCXE motif in a given protein in terms of a structure–function relationship appears to be high. This is also supported by the experimentally validated interaction between RB and human D-type cyclins known to contain the LXCXE sequence (Dowdy et al. 1993; Ewen et al. 1993).

**Future perspectives**

We therefore propose that the increase in cell proliferation seen under the conditions of embryonic development and neoplastic transformation is partially due to intracellular/intracrine growth regulatory mechanisms. Furthermore, we suggest that the administration of synthetic peptides that interfere with hormone/growth factor subunits and their receptors might emerge as a useful and specific approach in the future treatment of cancer. Since we have shown that insulin and viral oncoproteins share the RB-binding motif LXCXE, the same complementary peptide predicted by us (Wendtner & Radulescu, 1992) to block tumour virus-induced oncogenesis may also inhibit insulin-mediated cell growth stimulation. Hence, a single agent would intercept two different messages known to promote cell proliferation. We further infer that it might even be possible to design complementary peptides that specifically block the hormone/growth factor subunit responsible for growth control while leaving intact the region in charge of physiologically important metabolic effects. Past experience has shown that drugs can specifically reverse pathological conditions by targeting molecules that are both physiologically relevant and overexpressed during states of disease. The prerequisite for a successful outcome in this type of approach has been the compliance with the dose–effect principle. In support of this inference is, for example, the well-established therapeutic use of calcium channel blockers and β-adrenergic receptor antagonists in the treatment of arterial hypertension.

Our proposal of constructing peptides complementary to the LXCXE motif family in a number of proteins with oncogenic potential and using these compounds as specific anti-neoplastic agents in the clinical setting is in line with increasing efforts to develop structure-based strategies in the treatment of disease (Hale et al. 1988; Riechmann et al. 1988; Huse et al. 1989; Kuntz, 1992; Perutz, 1992) as well as with the recent focus on intracellular proteins as targets for therapeutic drug design (Brugge, 1993). Brugge pointed out that ‘... the legitimacy of targeting signaling pathways for therapeutic intervention has been demonstrated by the immunosuppressive drugs, cyclosporin A and FK506. Both of these drugs bind to calcineurin, a critical intracellular component of the T cell activation pathway that regulates the T-cell specific transcription factor, NF-AT. Although these drugs were discovered fortuitously, they prove that intervention in intracellular signaling pathways can effectively alter the course of a disease.’

**REFERENCES**


