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The Effects of Hypoxia on Human Cytotrophoblasts

By Van M. Hoang

Adviser: Professor Susan J. Fisher

Abstract: Once a blastocyst adheres to the uterus, the continued success of human pregnancy depends on the process whereby cytotrophoblasts (the specialized epithelial cells of the placenta) differentiate and invade the uterus and its blood vessels. Arterial invasion is particularly unusual and important. Cytotrophoblasts replace the maternal cells that originally formed the vessel wall. This process increases the vessel diameter by severalfold and enhances blood flow to the placenta. In an interesting feedback mechanism, the supply of maternal arterial blood (*e.g.*, oxygen tension) regulates the balance between cytotrophoblast proliferation and differentiation/invasion. Preeclampsia is a serious maternal vascular disorder that can occur during pregnancy. This syndrome adversely affects both mother (by altering vascular function) and fetus (by causing intrauterine growth retardation). Because preeclampsia is the leading cause of maternal death in developed countries and is associated with a fivefold increase in perinatal infant mortality, it continues to be a major health problem. This syndrome is associated with abnormal cytotrophoblast differentiation, shallow uterine arteriole invasion, and consequently, placental hypoxia. Here we examined the human cytotrophoblast proteome and studied the effects of decreased oxygen tension *in vitro* on the global cytotrophoblast protein repertoire via two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Hypoxia exerted a discrete effect upon the

cytotrophoplast proteins. The abundance of most proteins remained unaffected. Matrix assisted laser desorption/ionization time-of-flight/postsource decay analyses identified two antioxidants, peroxiredoxin and manganese superoxide dismutase, as being downregulated by hypoxia. Annexin II, tropomyosin, and the glycolytic enzymes triosephosphate isomerase and phosphoglycerate mutase were upregulated by hypoxia. Additionally, we discovered that although the levels of 14-3-3 epsilon were not affected by decreased oxygen tension, its localization in cytotrophoblasts was. These studies provided new information on the human cytotrophoblast protein repertoire and insight into pathways that maybe involved in cytotrophoblast response to changing oxygen levels.

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Chapter 1: Introduction

Implantation

The initial stages of preimplantation development, from zygote to morula, occur as the embryo encased within the non-adhesive zona pellucida transits the fallopian tube. The morula reaches the uterine cavity approximately two to three days postconception. Within 72 hours, the embryo enters the blastocyst stage and hatches from the zona, exposing its outer covering of trophoblasts. The few human embryos that have been recovered after maturation *in vivo* appear to be covered by multinucleate (syncytial) trophoblasts (Hertig and Rock, 1973), whereas at least some embryos that develop *in vitro* are covered by mononuclear cytotrophoblasts.

Implantation occurs around the sixth to seventh day postconception. Analogous to events in several primate species (Enders and Lopata, 1999) and rodents (Enders and Schlafke, 1969), human implantation is likely to occur in at least three stages. Initial adhesion of the blastocyst to the uterine wall, termed apposition, is probably unstable, i.e. the blastocyst can be easily dislodged. Apposition is characterized by interdigitation of the microvilli that compose the apical surface of syncytiotrophoblasts with microprotrusions from the apical surface of the luminal epithelium, known as pinopodes. Whether sufficient shear stress is exerted to permit rolling and tethering, as in leukocyte adhesion (Lawrence et al., 1995), is not known. Most commonly apposition, and consequently implantation, occur in the upper posterior (fundal) aspect of the uterus. During stable adhesion the physical interaction between the trophectoderm of the blastocyst and the luminal epithelium of the uterus strengthens. Penetration of the uterine

epithelium occurs shortly thereafter. By that time the blastocyst is oriented with its embryonic pole attached to the uterine wall (Hertig et al., 1959).

Human implantation is classified as intrusive (invasive) and interstitial (McMaster et al., 1994; Pijnenborg et al., 1981). Trophoblast cells rapidly invade the basal lamina that underlies the uterine epithelium. By day 10 postconception the blastocyst is completely embedded in subepithelial, interstitial stromal tissue, the uterine epithelium regrows over the implantation site, and mononuclear cytotrophoblasts stream out of the trophoblastic shell, further invading the uterus (Benirschke and Kaufmann, 1991).

Successful implantation is the end result of a complex molecular dialogue between a receptive, hormonally primed uterus and a mature, activated blastocyst. Timing is critical and failure to synchronize the component processes interrupts the dialogue. For example, an acceleration or delay in either uterine maturation or blastocyst activation prevents implantation. Uterine receptivity is defined as the temporal window during endometrial maturation that allows the blastocyst to implant (Psychoyos, 1986). Assisted reproductive techniques involving the transfer of human embryos back into the uterine cavity have identified days 20 to 24 of a regular 28-day menstrual cycle as the optimal period for human implantation (Bergh and Navot, 1992). Markers for uterine receptivity include gross histological changes (the endometrium becomes more vascular and edematous, endometrial glands display enhanced secretory activity) and alterations in endometrial cell surface properties (the luminal surface of endometrial epithelial cells develops pinopodes and shows evidence of electrophysiological changes) (Nikas and Psychoyos, 1997).

Of the multiple signals that synchronize blastocyst and uterine development, the role of steroid hormones is best understood. Human implantation requires a burst of preovulatory estradiol-17β that stimulates proliferation and differentiation of uterine glandular epithelia; continued progesterone production by the corpus luteum stimulates proliferation and differentiation of stromal cells. Downstream effectors of steroid hormone actions include peptide hormones, growth factors, and cytokines in the uterine milieu. For example, progesterone regulates calcitonin expression, which is detected in the rat uterine epithelium during the window of implantation (Ding et al., 1994). Growth factors that are expressed in the mouse uterus during the peri-implantation period include many of the epidermal growth factor family members. Interestingly, expression of heparin-binding epidermal growth factor is restricted to the luminal epithelium surrounding the blastocyst just prior to implantation (Das et al., 1994; Leach et al., 1999). Likewise, a burst of leukemia inhibitory factor expression in endometrial glands precedes implantation of the mouse blastocyst (Bhatt et al., 1991). Analyses of leukemia inhibitory factor -/- mice show that this molecule is required for embryo attachment and uterine decidualization. Unexpectedly, interleukin-11 interactions with the interleukin-11 receptor alpha chain are also required for murine decidualization (Robb et al., 1998).

The blastocyst is an active participant in the process that readies the uterus for implantation (Paria et al., 1993). Mechanisms that activate the blastocyst include exposure to catecholestrogens, a class of estrogen metabolites (Paria et al., 1998). Culture medium of preimplantation embryos contains a myriad of bioactive substances with potential autocrine and/or paracrine effects. These include transforming growth factor-α and -β, platelet-derived growth factor, insulin-like growth factor-II, colony-

stimulating factor-1, interleukin-1 and -6, leukemia inhibitory factor, prostaglandin E_2 and platelet-activating factor as well as receptors for colony stimulating factor-1, epidermal growth factor, and leukemia inhibitory factor (Stewart and Cullinan, 1997).

The clearest evidence of cross-talk between the blastocyst and the uterus comes from studies of the expression of epidermal growth factor family members in a murine model of hormonally delayed implantation. During delay, uterine heparin-binding epidermal growth factor expression is not induced, even when the blastocyst is juxtaposed to the uterine lining. Upon injection of estrogen, the implantation process resumes with blastocyst activation, and uterine heparin-binding epidermal growth factor expression is rapidly upregulated at the site of blastocyst apposition (Das et al., 1994). Completing the loop, peri-implantation-stage embryos express epidermal growth factor receptors (ErbB1, -B2 and -B4) and heparin sulfate proteoglycans that interact with epidermal growth factor-like ligands; addition of heparin-binding epidermal growth factor to cultured embryos stimulates their proliferation, as well as maturation evidenced by accelerated zona hatching and trophoblast outgrowth (Das et al., 1994; Jun, 2000). At least a portion of this scenario is probably applicable to human implantation, since heparin-binding epidermal growth factor appears to have similar effects on human embryos *in vitro* (Martin et al., 1998). Interestingly, chorionic gonadotropin alters endometrial differentiation in the baboon (e.g., upregulates glycodelin expression), evidence that the primate blastocyst may also communicate with the uterus prior to implantation (Fazleabas et al., 1999).

Implantation in the mouse requires the biosynthesis of prostaglandins. Cyclooxygenase, the rate-limiting enzyme in the conversion of arachidonic acid into

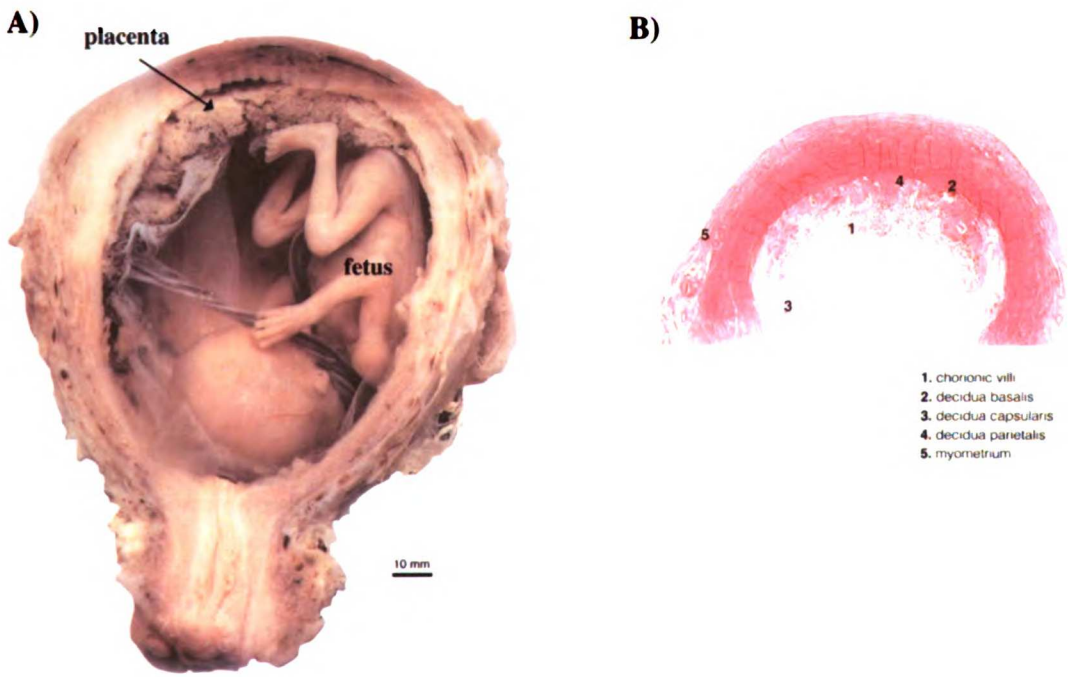
prostaglandin H_2, exists in two isoforms: constitutive (cyclooxygenase-1) and inducible (cyclooxygenase-2). Endometrial cyclooxygenase-1 expression is responsive to both progesterone and estradiol-17β; levels fall precipitously around the time of implantation (Chakraborty et al., 1996; Marions and Danielsson, 1999). In contrast, cyclooxygenase-2 expression, which is not affected by steroid hormones, is restricted to the site of implantation and depends on the presence of an activated blastocyst (Chakraborty et al., 1996; Lim et al., 1997a). There is evidence that the same pattern may exist in humans: interleukin-1β, which has been detected in the conditioned medium of human embryos (Sheth et al., 1991), induces cyclooxygenase-2 expression in cultured endometrial stromal cells (Huang et al., 1998). Recent work shows that prostacyclin derived from nuclear cyclooxygenase-2 interacts with peroxisome proliferator activated receptor-δ (Lim et al., 1999a). This interaction is likely a critical component of the downstream effector pathway, because peroxisome proliferator activated receptor-δ -/- mice die at mid-gestation due to placental defects (Barak et al., 1999). Interestingly, other molecules that play important roles in implantation have also been linked to prostaglandins. These include the abdominal B-like homeobox genes (e.g., HOXA-10 and HOXA-11) previously thought to be associated only with fetal organ development. HOXA-10-deficient mice have abnormalities in decidualization and implantation (Benson et al., 1996). These defects have been linked to aberrations in progesterone-regulated expression of two prostaglandin E_2 receptor subtypes, EP3 and EP4, in the uterine stroma (Lim et al., 1999b). Finally, HOXA-10 expression in the human uterus, which is regulated by both progesterone and estradiol-17β, peaks during the implantation window (Ma et al., 1998; Taylor et al., 1997). Together, these data offer the first potential

molecular explanation for the changes in vascular permeability that are one of the first signs of implantation in many species.

Once human implantation begins, a comparatively brief interval of stable attachment is soon followed by a much longer period during which trophoblast cells exhibit the unusual tumor-like behavior that enables them to invade the uterine luminal epithelium and underlying endometrial stroma. As in other biological systems in which stable adhesion is followed by invasion (e.g., leukocyte and tumor extravasation), strategic modulation of adhesion molecule and proteinase expression is likely a key element of the strategy trophoblasts use to enter the uterine wall. Because most of our information comes from studies of specimens obtained mid-way through the first trimester, whether the process of cytotrophoblast invasion of the uterine wall during the first half of pregnancy recapitulates the mechanisms involved in initial invasion during implantation is an important unresolved question.

Placental Growth and Differentiation in Normal Pregnancy

After implantation, in order to maintain a pregnancy, the placenta, a disk-shaped organ, must successfully grow and develop (Figure 1). Placentation is the result of the growth and differentiation of cytotrophoblasts, the specialized epithelial cells of the placenta. A diagram of this process is shown in Figure 2A. During normal placentation, CTB stem cells undergo one of two different fates (Fisher and Damsky, 1993). First, in floating villi, they detach from their underlying basement membrane and fuse to form syncytiotrophoblast that cover the surface of floating villi. The surface of syncytiotrophoblast lines the intervillous space where gas, waste, and nutrient exchange



Week 12 Gestation

Figure 1. A) The placenta and fetus *in utero*. B) A transverse section of the upper portion of the pregnant uterus.

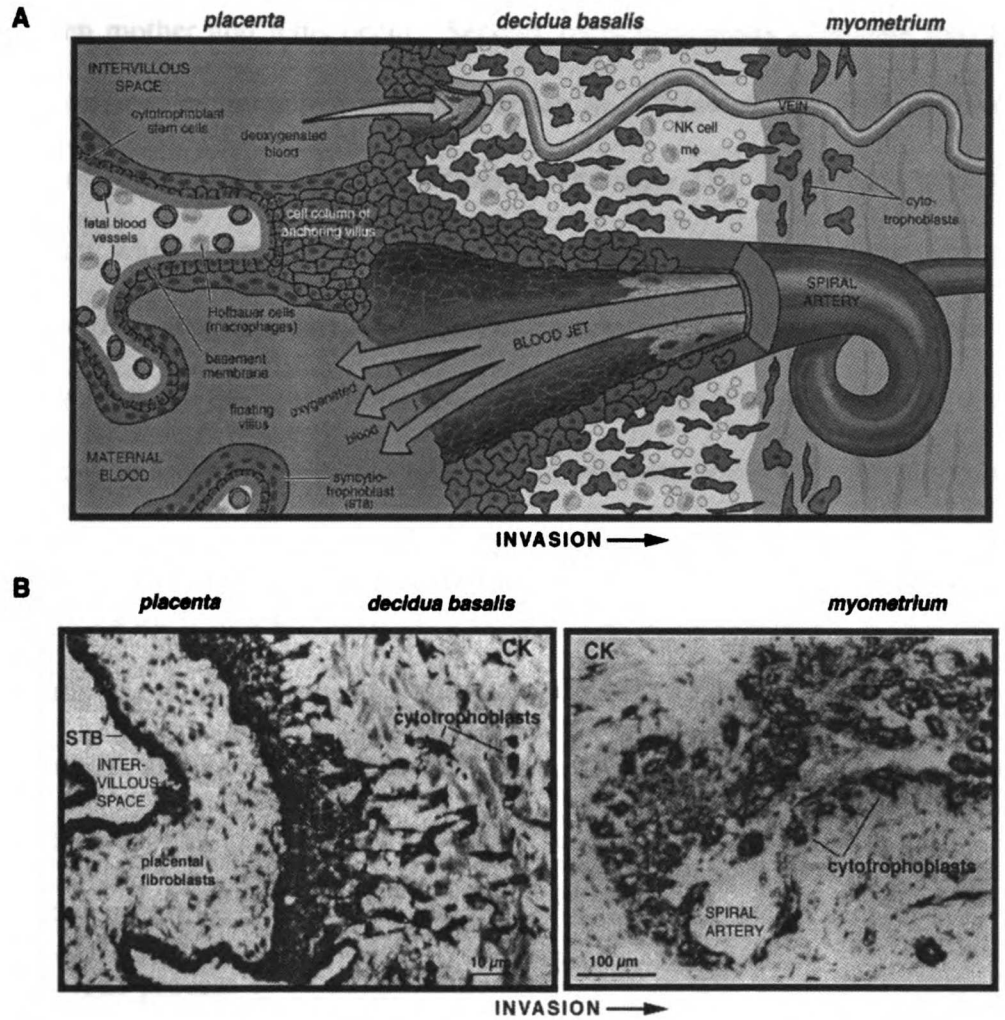


FIGURE 2: Human placental cytotrophoblast differentiation *in vivo*. (A) Diagram depicts the human maternal-fetal interface at mid-gestation. The specialized epithelial cells of the placenta (cytotrophoblasts) differentiate along one of two pathways. In the first, they detach from the underlying basement membrane and fuse to form syncytiotrophoblasts (STB) that cover the surfaces of the villi. In the second pathway, they aggregate into columns of nonpolarized cells that bridge the gap between the fetal and maternal tissues. Individual cytotrophoblasts that emerge from these columns differentiate into tumor-like cells that invade the uterine wall. Invasive cells also breach maternal blood vessels, where they replace the endothelial lining of arteries and, to a lesser extent, veins. In this location these placental cells upregulate the expression of adhesion molecules and receptor tyrosine kinases that are usually found on the endothelial cell surface. In the end, cytotrophoblast invasion anchors the placenta to the uterine wall and diverts the flow of maternal blood to the placenta (see arrows). (B) Tissue sections of the maternal-fetal interface cut from a biopsy of the area diagrammed in (A). Cytokeratin (CK) staining identifies the syncytio- (STB) and cytotrophoblasts. The photomicrograph on the left shows a cytotrophoblast column that attaches a villus to the uterine wall and invasive cytotrophoblasts that migrate from the column into the decidua. The photomicrograph on the right shows cytotrophoblasts that replaced the maternal cells in the wall of a uterine spiral arteriole. Both tissue sections were adapted from work in done in our laboratory (Zhou et al., 1993).

between mother and fetus occur. Second, cytotrophoblasts aggregate into columns of nonpolarized, mononuclear cells that attach to and invade the wall of the uterus (see diagram in Figure 2A and photomicrograph in Figure 2B). In addition to this interstitial invasion, which normally encompasses the entire endometrium and the inner third of the myometrium, they also carry out endovascular invasion, a process that diverts the flow of uterine blood to the intervillous space of the placenta (Pijnenborg et al., 1981). Once cytotrophoblasts have migrated into the endometrium, many of them invade its arteries, eventually replacing the endothelial cells of these vessels. Invasion of the arteries transforms them into low resistance vessels with large diameters, which allows for the increase of blood supply to the intervillous space to support the developing conceptus (Figure 2A and 2B). Hence, the mature human placenta is classified as hemochorial. Blood (hemo) is in direct contact with the placental portion of the extra-embryonic membrane (chorio).

The process of cytotrophoblast invasion is equally unusual at a molecular level. The invading cells extensively modulate their adhesion molecule expression in a stepwise fashion. Particularly striking is the reduced staining for adhesion receptors characteristic of polarized cytotrophoblast stem cells (integrin $\alpha 6\beta 4$ and epithelial cadherin) and the onset of expression of adhesion receptors characteristic of endothelium, including cadherins (vascular endothelial cadherin and cadherin-11), IgG-family receptors (vascular cell adhesion molecule-1, platelet-endothelial cell adhesion molecule-1 and Mel-CAM), and integrins ($\alpha V\beta 3$ and $\alpha 1$) (Damsky and Fisher, 1998).

In addition to allowing cytotrophoblasts that line maternal vessels to masquerade as vascular cells, many of these receptors also play important roles in invasion. Analysis

of the effects of adding function-perturbing antibodies to an *in vitro* model of cytotrophoblast invasion reveals a delicate balance. For example, integrins αVβ3 and α1β1 promote cytotrophoblast invasion, but α5β1 restrains it (Damsky et al., 1994). *In vivo*, trophoblast invasion is likely influenced by uterine extracellular matrix, ECM, components (e.g., laminin, fibronectin, and osteopontin) that are ligands for trophoblast integrins (Lessey, 1998; Lessey and Arnold, 1998). Adhesion molecule expression is also extensively modulated during decidualization. Analysis of human embryos that developed *in vitro* suggests that maturation is associated with the expression of at least a subset of the adhesion receptors that are detected in association with cytotrophoblasts later in gestation (Campbell et al., 1995).

Invading cytotrophoblasts also tightly regulate their proteinase repertoire (Huppertz et al., 1998). Of particular functional importance is their ability to express and activate matrix metalloproteinase-9, a major regulator of cytotrophoblast invasiveness *in vitro* (Librach et al., 1991). Interestingly, matrix metalloproteinase-9 deficient mice have a substantial reduction in litter size, suggesting defective placentation (Dubois et al., 2000). The simultaneous upregulation of tissue inhibitor of metalloproteinase-3 expression is another example of the balancing mechanisms that hold cytotrophoblast invasion in check (Bass et al., 1997). Invasion also involves maternal expression of matrix metalloproteinase family members. Studies in genetically engineered mice indicate that decidual expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases affects the extent of trophoblast invasion (Alexander et al., 1996), and their expression in human decidua suggests a similarly important role (Riley et al., 1999). Other studies in mice suggest that cathepsins B and L may play an important role in

invasion (Afonso et al., 1997). In fact, the placenta expresses an interesting set of these proteinases, including novel cysteine proteases (Bogyo et al., 2000). Although trophoblast-associated urokinase-type plasminogen activator is probably not involved in invasion, it, like the aforementioned adhesion molecules, may play a role in vascular mimicry (Queenan et al., 1987). A subset of these proteinases and inhibitors (e.g., matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-3) is produced by human embryos *in vitro*, suggesting that they could function from implantation onward.

The specific temporal and spatial expression of several growth factors and cytokines within the uterus (e.g., leukemia inhibitory factor (Cullinan et al., 1996), interleukin-1 and its receptors (Simon et al., 1996), insulin-like growth factor-1 and -2 and their binding proteins (Giudice and Irwin, 1999), colony stimulating factor-1 (Cohen et al., 1997), transforming growth factor-α (Slowey et al., 1994; Taga et al., 1996) and -β (Godkin and Dore, 1998)) suggests that they could play a modulatory role. In many cases the functional influences are inferred, but in others direct experimental evidence exists. For example, leukemia inhibitory factor regulates matrix metalloproteinase-9 expression in mouse blastocysts (Harvey et al., 1995), as does interleukin-1 in human cytotrophoblasts (Librach et al., 1994). It is interesting that interleukin-1 levels in embryo culture supernatants have been associated with reproductive success during *in vitro* fertilization (Sheth et al., 1991). Physiological regulators may also be particularly important. For example, oxygen tension controls some aspects of differentiation in cytotrophoblasts, including integrin α1 expression (Genbacev et al., 1997).

The maintenance of early pregnancy is inextricably linked with growth and differentiation of the cells that compose the extra-embryonic membranes. In accord with

its critical functions, the trophoblast is the first lineage specified during development. Much has recently been learned, sometimes inadvertently, from gene deletion studies in mice about the transcription factors that regulate these early fate decisions. For example it has recently been reported that levels of Oct-4 determine whether mouse embryonic stem cells differentiate into trophectoderm which gives rise to trophoblasts, mesoderm, or endoderm (Niwa et al., 2000). Also, Mash2, a mammalian homologue of *Drosophila* achaete/scute complex genes, is involved in the maintenance of the murine trophoblast stem cell population, a critical determinant of growth potential. Commitment to the differentiation pathway that leads to the invasive population of trophoblast cells is characterized by downregulation of the expression of inhibitor of DNA binding proteins (Ids) (Jen et al., 1997), the mSNA zinc finger transcription factor (Nakayama et al., 1998), and Oct-4 (Palmieri et al., 1994). Upregulated expression of the positive regulator Hand1 is required to complete this differentiation process (Firulli et al., 1998; Riley et al., 1998). Glial cells missing-1, a novel factor that controls differentiation in the *Drosophila* nervous system, regulates the pathway that gives rise to murine multinucleate trophoblasts, a population that is in some ways analogous to human syncytiotrophoblasts (Anson-Cartwright et al., 2000). Conversely, lack of expression of estrogen-receptor-related receptor-β is associated with an overabundance of invasive cells (Luo et al., 1997).

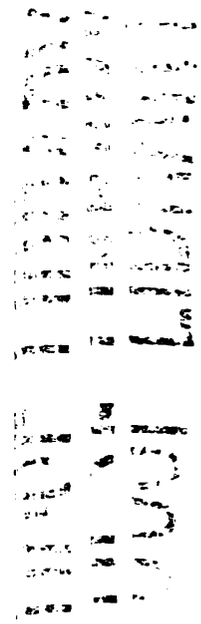
Although there are numerous differences between the mouse and the human placenta at a morphological level, many of the same transcriptional regulators that control murine placentation are expressed by human trophoblasts (Janatpour et al., 1999). Functional evidence supports the hypothesis that they play an important role.

For example, Id-2 regulates aspects of cytotrophoblast differentiation along the invasive pathway (Janatpour et al., 2000), and Oct-4 overexpression silences expression of the beta subunit of human chorionic gonadotropin (Liu and Roberts, 1996). Finally, it is interesting to note that cytotrophoblasts are the only cells that express glial cells missing-1; the functional consequences of this observation are currently being studied in our laboratory.

Growth factors also play interesting roles, often in a paracrine manner, supporting the concept that epithelial–mesenchymal interactions are important for placental development. Mice that carry homozygous mutations in the scatter factor/hepatocyte growth factor gene show defects in trophoblast differentiation attributable to absence of this signal, which is normally derived from the underlying allantois. Mice lacking the hepatocyte growth factor receptor, c-met, die during development from placental insufficiency secondary to abnormal placental morphogenesis (Bladt et al., 1995). In the human, mesenchymal cells in the stromal cores of chorionic villi produce hepatocyte growth factor, cytotrophoblasts express c-met (Bladt et al., 1995; Clark et al., 1996; Kilby et al., 1996), and hepatocyte growth factor enhances cytotrophoblast invasion *in vitro* (Nasu et al., 1999). Strong evidence also supports the role of fibroblast growth factors family members. The expression pattern of certain of the factors (e.g., fibroblast growth factor-4) and receptors (e.g., fibroblast growth factor receptor-2), as well as their null phenotypes (e.g., peri-implantation lethality in mice) suggests that the trophoblast is an important target. In fact, trophoblast stem cells were derived by plating blastocysts or early postimplantation trophoblasts on a fibroblast feeder layer in the presence of fibroblast growth factor-4. Growth factor

withdrawal resulted in differentiation (Tanaka et al., 1998).

One of the most interesting placental functions is the regulation of the maternal immune response such that the fetal hemi-allograft is tolerated during pregnancy. Syncytiotrophoblasts and invasive cytotrophoblasts are presumed to be essential to this unique phenomenon because they lie at the maternal-fetal interface, where they are in direct contact with cells of the maternal immune system. Several studies suggest that none of the trophoblast subpopulations express class II molecules (Bulmer and Johnson, 1985; Redman, 1983). Surprisingly, cytotrophoblasts upregulate expression of the class Ib major histocompatibility complex protein (HLA-G) as they differentiate and invade the uterus (Ellis et al., 1990; Kovats et al., 1990; McMaster et al., 1998). This pattern of expression, together with the fact that HLA-G exhibits limited polymorphism (Bainbridge et al., 1999), suggests it has an important function in pregnancy. But the exact mechanisms involved remain enigmatic. One obstacle has been the identification of HLA-G receptor(s) on relevant cells, a controversial area (Bainbridge et al., 1999).



Within the uterine wall, cytotrophoblasts that express HLA-G come in contact with maternal lymphocytes, which are abundant in the uterus during early pregnancy. Although estimates vary, a minimum of 10% to 15% of all cells found in the decidua are leukocytes, many of which are found in large lymphoid clusters (Mincheva-Nilsson et al., 1994). Although it is clear that immune recognition of paternally-derived antigens occurs during pregnancy (Tafuri et al., 1995), cytotoxicity against trophoblast must be inhibited. The factors responsible for this localized immunosuppression are unclear, but likely include cytotrophoblast-derived interleukin-10, a cytokine that inhibits alloresponses in mixed lymphocyte reactions (Roth et al., 1996). Interestingly, steroid

hormones, including progesterone, can have similar effects (Pavia et al., 1979). The complement system must also be involved, because deletion of the complement regulator, Crry, leads to fetal loss secondary to placental inflammation in mice (Xu et al., 2000). Finally, pharmacological data, also in the murine system, suggest that trophoblasts express an enzyme, indoleamine 2,3-dioxygenase, that rapidly degrades tryptophan, which is essential for T-cell activation (Munn et al., 1998). Whether this same mechanism operates in humans is not yet known, although syncytiotrophoblasts express indoleamine 2,3-dioxygenase (Kamimura et al., 1991) and systemic tryptophan levels fall during pregnancy (Schrocksnadel et al., 1996).

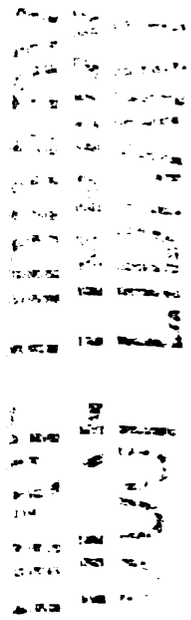
In summary, cytotrophoblast differentiation and placental development are regulated by a multitude of factors. These include transcription factors, proteases, cytokines, adhesion molecules, growth facors, and oxygen tension. Determining molecules and pathways that result in normal placentation gives insights into mechanisms involved in pregnancy complications that stem from abnormal placental development which is discussed in the following section.

Pregnancy Complications

Normal human placentation shares elements of the developmental program that is executed during embryonic organogenesis. However, a higher-order requirement is imposed on placental development because this organ not only develops first, but also serves as the structural and functional interface between the mother and the embryo/fetus. At a structural level, cytotrophoblasts that emanate from anchoring villi invade to a depth that securely anchors the placenta to the uterine wall. During this

invasion process the cells also form stable connections with the maternal vasculature, a process that diverts the flow of uterine arterial blood to the placenta and creates a pathway for venous return.

At a functional level, the placenta must integrate maternal and fetal physiology, immunology, and endocrinology. For example, nutrient, gas, and waste exchange take place in the intervillous space, which is lined by the syncytiotrophoblasts that cover the floating villi. The intriguing possibility exists that complications that arise relatively late during pregnancy actually reflect errors that occurred much earlier in the program that governs placental development. The correlative — that such errors could be detected and ameliorative or corrective action taken before pregnancy outcome is compromised — is a major challenge facing maternal-fetal medicine.



Biopsy of the placenta and uterus at the time of delivery has allowed microscopic assessment of the floating and anchoring villi that compose the maternal-fetal interface in a variety of pregnancy complications. With regard to floating villi, intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with fewer capillary loops and branches in the villus cores, and with fibrin deposition on the syncytial surface (Krebs et al., 1996). The maternal vasculature is also important, as directed vascular expression of the receptor for thromboxane A_2, a smooth muscle cell constrictor and mitogen, leads to (murine) intrauterine growth retardation (Rocca et al., 2000). Heritable coagulopathies lead to (human) intrauterine growth retardation and other serious pregnancy complications (Lockwood, 1999).

Maternal cigarette smoking has a severe negative effect on all aspects of pregnancy, from conception to birth, when many affected infants show evidence of

intrauterine growth retardation. Given the known actions of nicotine and hypoxia, it is not surprising that the placentas of women who smoke show dramatic changes in morphology when compared to those of gestation-matched control placentas obtained from women who do not smoke (Shiverick and Salafia, 1999). The fetal blood vessels within the villus mesenchymal cores also develop adaptations, principally increased density (Pfarrer et al., 1999), and areas of the syncytium thin dramatically (Genbacev et al., 1999a). Clearly, compromised syncytiotrophoblast function is associated with impaired fetal growth.

With regard to anchoring villi, cytotrophoblast invasion to the proper depth is a major factor in determining pregnancy outcome. Excessive invasion can lead to deficient development of the decidua with abnormally firm attachment of the placenta directly onto the myometrium (placenta accreta), to extension into the myometrium (placenta increta), or to invasion through the myometrium to the uterine serosa and even into adjacent organs (placenta percreta). Despite improvements in diagnosis and clinical management, such disorders of placentation are still associated with significant intrapartum maternal morbidity and mortality, due primarily to hemorrhage (O'Brien et al., 1996). Inadequate invasion has been implicated in the pathophysiology of intrauterine growth retardation and preeclampsia which is discussed in the following section.

Preeclampsia

Preeclampsia is the leading cause of maternal mortality in the industrialized world and increases perinatal mortality fivefold. The clinical diagnosis includes the

sudden onset of hypertension, blood pressure greater than 140/90 mm Hg, the appearance of proteinuria, over 300 mg in a 24 hour collection, and edema. The symptoms usually develop after 20 weeks of gestation and onset and progression of the disease are unpredictable. If not treated, seizures (known as eclampsia), coma, and death can follow.

There are many risk factors associated with preeclampsia. Although preeclampsia is thought of as a disease of first pregnancies, a change in partner with subsequent pregnancies is associated with the loss of the protective effect of multiparity; similarly, artificial donor insemination is also reported to result in increased risk of preeclampsia (Dekker, 1999). The use of contraceptive devices which prevent exposure to sperm also increases the risk of preeclampsia (Klonoff-Cohen et al., 1989). These epidemiological studies suggest that maladaptation to a paternal factor may play a role in the development of preeclampsia. Genetics can also be a risk factor in the development of preeclampsia, reviewed in (Morgan and Ward, 1999). The rate of preeclampsia is higher in mothers, sisters, daughters, and granddaughters of women with preeclampsia, although the mode of inheritance is unclear.

The clinical symptoms of preeclampsia are only a small component of a multisystem disease. Because of the importance of the maternal immune system during normal pregnancy, immune maladaptation is reported to be involved in preeclampsia. Many observations point to the involvement of the immune system, including exposure to paternal antigens as described previously. Levels of tumor necrosis factor-α (Meekins et al., 1994a) and interleukin-6 (Vince et al., 1995) are increased in blood samples from preeclamptic patients. Also, there is increased staining for interleukin-2 in decidua of

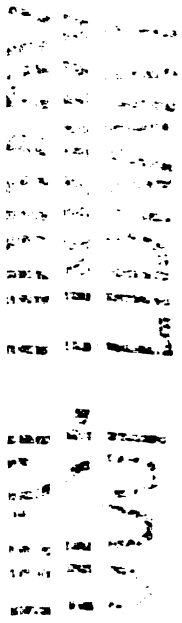
women with preeclampsia (Hara et al., 1995), and HLA-G expression is downregulated on cytotrophoblasts in preeclampsia (Lim et al., 1997b).

Abnormal lipid metabolism is also believed to contribute to the development of preeclampsia. Serum triglyceride and free fatty acid concentrations are increased in pregnant women with preeclampsia relative to normal pregnant women (Hubel et al., 1996; Lorentzen et al., 1995; Vigne et al., 1997) while total cholesterol levels are not different. The increased levels of circulating free fatty acids are reported to be increased 15 to 20 weeks prior to the clinical symptoms of preeclampsia (Lorentzen et al., 1995).

An increasing body of data supports the hypothesis that many aspects of the disease process could be explained by alterations in the function of maternal vascular endothelium (Roberts and Redman, 1993), including an excessive inflammatory response (Redman et al., 1999). Changes in maternal vessel relaxation in response to a variety of vasoactive substances in preeclamptic women as compared to normal pregnant women have been described. For example, there is impaired endothelium-dependent relaxation in preeclamptic women in response to bradykinin (Ashworth et al., 1999) and acetylcholine (McCarthy et al., 1993). The converse question of whether sera from preeclamptic women have different effects on endothelium function than sera from normal pregnant women has also been investigated. For example, exposure of human umbilical vein endothelial cells to the plasma from preeclamptic patients changed the ecoisanoid secretion profile of these cells; increased secretion of prostacyclin, prostaglandin $F_2\alpha$, and 8-isoprostane was noted (de Groot et al., 1998). Results such as these suggest the presence of circulating factors present in preeclampsia which are either absent in normal pregnancies or modulated abnormally in preeclampsia. The origins of

such factors are debated, but likely sources include the placenta (Redman, 1991).

Preeclampsia is believed to originate from a placental disorder, since it occurs in complete molar pregnancies in which there is trophoblast, but no fetal tissue (Goldstein and Berkowitz, 1994). Also, delivery of the fetus and placenta remains the only effective treatment. Although the etiology of preeclampsia is unknown, the characteristic pathological lesion is variably shallow interstitial cytotrophoblast invasion and, more consistently, restricted endovascular invasion (Brosens, 1977; Genbacev et al., 1999b; Meekins et al., 1994b; Zhou et al., 1993). Immunolocalization studies of biopsy specimens suggest a focal molecular lesion that affects a specific aspect of placental development (Figure 3). Normally cytotrophoblasts that invade uterine vessels switch their adhesion molecule repertoire to resemble that of vascular cells, but in preeclampsia many aspects of this program fail (Zhou et al., 1993). In turn, the uterine arterioles remain as small-bore, high-resistance vessels that cannot adequately respond to the ever-increasing fetal demands for blood flow. Ultrasound Doppler techniques have shown that uteroplacental blood flow is reduced in preeclampsia (Campbell et al., 1986; Harrington et al., 1997). Determining the consequences of reduced placental perfusion and how they ultimately lead to the clinical characteristics of preeclampsia is a major challenge.



In Vitro Models

In human diseases, where direct experimentation is usually not possible, we have utilized *in vitro* culture models to better understand normal human cytotrophoblast differentiation (Figure 4). Cytotrophoblasts isolated from placentas of women with

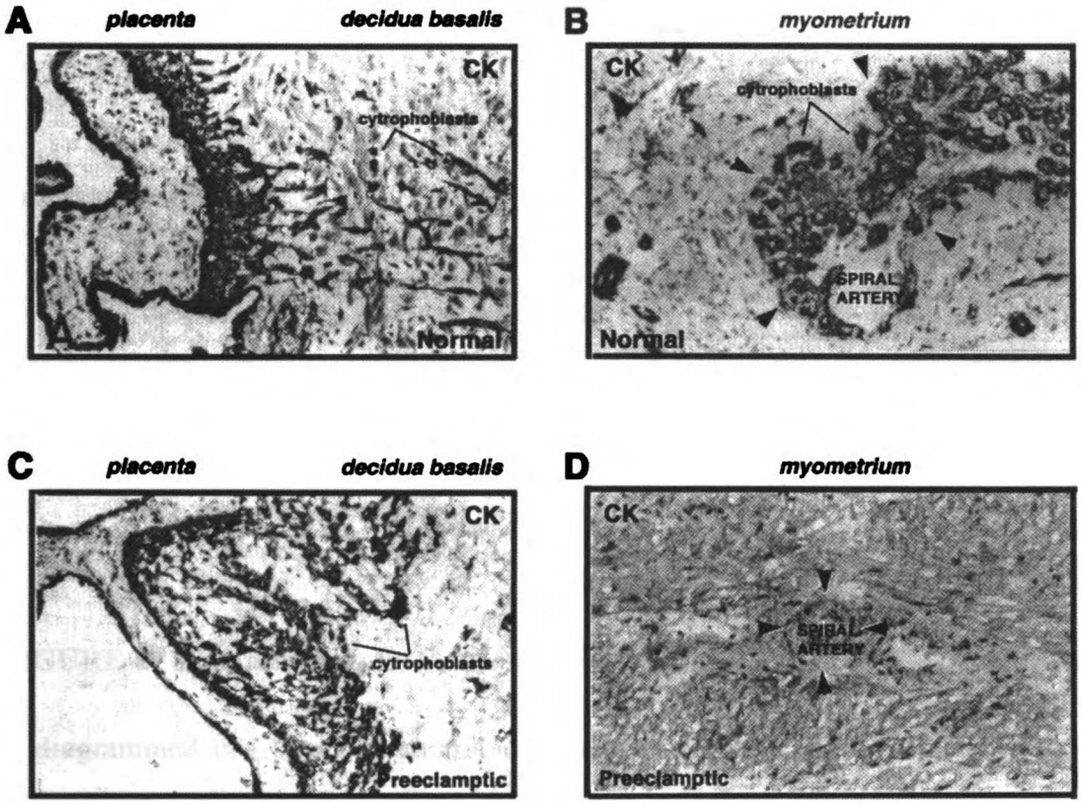


FIGURE 3. Placental bed biopsies from preeclamptic pregnancies reveal reduced cytotrophoblast invasion of the uterus and its spiral arteries. (A) and (B) During the course of a normal pregnancy, cytotrophoblasts invasion occurs through the decidua and the myometrium. As previously shown, the maternal uterine arteries are also replaced by these invading cells. (C) and (D) In preeclamptic pregnancies, there is reduced invasion of the uterus and its resident arteries. Due to decreased invasion by cytotrophoblasts, the radii of the arteries remain constricted in preeclampsia as compared to normal pregnancies resulting in decreased blood flow to the placenta. Cytokeratin (CK) staining identifies syncytiotrophoblast and cytotrophoblasts. Arrowheads identify the spiral arteries in the myometrium. This figure is modified from (Zhou et al., 1993).

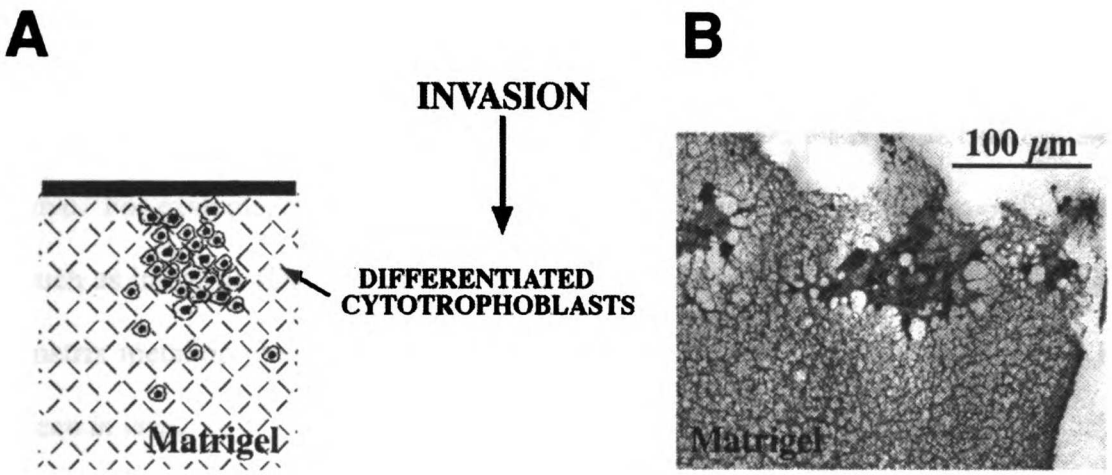


FIGURE 4. Human placental cytotrophoblast differentiation *in vitro*. This process is diagrammed in (A) and a photomicrograph of a typical culture is shown in (B). Cytotrophoblast stem cells that are isolated from human placentas and plated on a Matrigel (MG) substrate undergo the same differentiation process that is observed during uterine invasion *in situ*. By 48 h they are embedded within the substrate.

uncomplicated pregnancies are maintained under standard culture conditions (20% O_2) on Matrigel, a tumor extract that contains basement membrane components. This induces the cells to enter the differentiation pathway that leads to uterine invasion *in vivo*. For example, cytotrophoblasts cultured on Matrigel express stage specific antigens such as integrins (Damsky et al., 1994; Fisher and Damsky, 1993; Zhou et al., 1997b), matrix metalloproteinase-9 (Librach et al., 1991), and HLA-G (McMaster et al., 1995) seen *in vivo*.

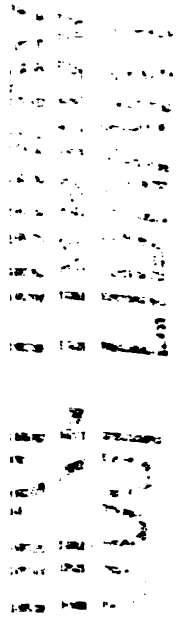
Tissue explants of chorionic villus are also used to study placental development. With this model, all cell types found in the placenta *in vivo* are present including syncytiotrophoblast and stem cell cytotrophoblasts. Culture of villus explants on Matrigel induces the formation of cytotrophoblast outgrowths that share many characteristics of anchoring villi (Genbacev et al., 1993; Genbacev et al., 1992).

Experiments in which normal cytotrophoblasts were maintained in hypoxia (2% O_2) reproduced many of the defects seen in preeclampsia. For example, cytotrophoblasts cultured under hypoxic conditions show reduced invasion, as measured by the ability to traverse a Matrigel-coated filter (Genbacev et al., 1996). Also, hypoxia abnormally modulates adhesion molecule expression such as the downregulation of integrin α1which correlates to what is seen in preeclampsia (Zhou et al., 1993; Zhou et al., 1997a). In addition, oxygen tension controls some aspects of placental growth and cytotrophoblast differentiation. Villus explants cultured in 2% O_2 show continued proliferation of cytotrophoblasts and impaired differentiation and invasion while those cultured in 20% O_2 showed differentiation of cytotrophoblasts (Genbacev et al., 1997). This corresponds to findings in preeclampsia of an increased number of proliferative

cytotrophoblasts (Redline and Patterson, 1995).

Because molecules released from the placenta have the potential to exert an effect on the maternal vasculature, factors secreted by cytotrophoblasts maintained under standard conditions or hypoxia have also been examined. Conditioned medium from hypoxic cultures increases vessel sensitivity to vasoconstriction (Gratton et al., manuscript in preparation); similar responses have been described in vessels isolated from preeclampsia patients (Aalkjaer et al., 1985). These results strengthen the linkage between placental hypoxia and preeclampsia.

In light of the current information about the relationship between reduced oxygen tension and pregnancy complications, we have been very interested in determining the effects of hypoxia on the cytotrophoblast protein repertoire. As discussed next, a proteomics-based approach to survey changes that occur in response to hypoxia was chosen. Knowing whether similar or different changes occur in preeclampsia is equally important for determining how the syndrome progresses.

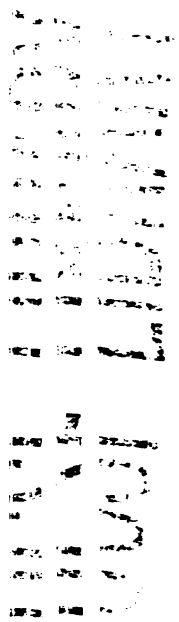


Proteomics

The rapid development and improvement of DNA technologies has led to the success of multiple genome projects. The completion of genome sequences from at least 18 organisms, with many more expected in the near future (Blackstock and Weir, 1999), has been followed by the realization that genomic sequences are not adequate to fully understand complex biological processes. The burgeoning of the field of proteomics is a product of this awareness.

Proteomics is broadly defined as the study of the protein complement of the genome. This wide encompassing term, however, is a field, just as genomics is. In general, the term is used to describe studies that seek to define and explore relative protein levels in order to characterize and define biological processes. Although in its infancy relative to genomics, this area of investigation is (arguably) among the fastest growing and exciting area of science today.

Proteomics studies are based on techniques designed to separate and rapidly identify proteins (Blackstock and Weir, 1999; Haynes et al., 1998; Hochstrasser, 1998). Methods used to separate complex protein mixtures include subcellular fractionation, chromatography, immunopurification, and two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Furthermore, many studies use combinations of these methods. For example, Edman sequencing and mass spectrometry are commonly used to determine protein sequence and identity. Here we used a combination of 2-D PAGE and mass spectrometry.



Genomic studies usually focus on one of two goals. In the first, the genome of an organism is sequenced. In the second, termed functional genomics, experiments are designed to identify differences, at the DNA level, that are induced by a wide variety of factors, either *in vitro* or *in vivo*. For example, pharmacogenomics deals with variability in drug efficacy and toxicity in different populations due to genetic polymorphisms (Evans and Relling, 1999). It has been reported that variations in the gene that encodes the serotonin (5-HT) receptor are implicated in the efficacy of treatment of schizophrenia with clozapine (Arranz et al., 1998). Also, recent attempts at mapping single nucleotide

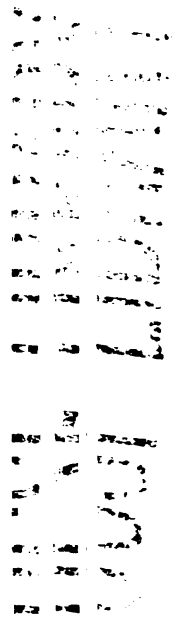
polymorphisms (SNPs), led to the discovery of a common polymorphism in *KCNE2* that is associated with drug-induced arrythmia (Sesti et al., 2000).

Likewise, proteomics approaches can be subdivided according to focus. Protein-expression mapping entails the production of reference maps of proteins expressed in a given system. Functional proteomics, includes studies that identify protein-protein interactions and changes in protein levels and modifications that are induced by various stimuli. As in functional genomics, the effects of a vast number of variables can be investigated in these studies.

Reference expression maps have been created of proteins isolated from a variety of sources. These include rat liver (Li et al., 1997), human bronchoalveolar lavage (Wattiez et al., 1999), murine mammary epithelial cells (Fialka et al., 1997), human reflex tear fluid (Molloy et al., 1997), placental mitochondria (Rabilloud et al., 1998), placental lysosome (Chataway et al., 1998), and *Saccharomyces cerevisiae* (Perrot et al., 1999). This systematic cataloging of proteins defines the normal or basal state of a biological system. As a result, comprehensive 2-D gel databases can be established that allow comparisons between normal and experimental or diseased states. In order to provide increased accessibility, these databases can be reached on the internet at sites such as http://www-lmmb.ncifcrf.gov/2dwgDB/2DgelDBtable.html and http://www.expasy.ch/ch2d/2d-index.html. As the information in these databases grows, so will their value. Eventually, they will provide indispensable information to scientists worldwide in identifying proteins and protein networks that play critical roles in normal and diseased states.

Researchers have also been using proteomics to study the regulated expression of proteins in a variety of systems. These studies are crucial to rapid identification of global changes induced by diverse stimuli. Examples of this application of proteomics techniques include the identification of proteins in cells that are phosphorylated upon stimulation with platelet-derived growth factor (Soskic et al., 1999), and the detection and identification of proteins induced by γ-interferon and interleukin-4 treatment of human renal carcinoma cells (Sullivan et al., 1997).

Finally, proteomics approaches are commonly used to identify protein-protein interactions. Identifying the protein partners in a complex is crucial to drawing structure-function correlation about individual members as well as the complex as a whole. For example, proteomics has been successfully utilized in the identification of proteins in various biological and signaling complexes such as the characterization of the subunits of the anaphase promoting complex (Zachariae et al., 1998), and the discovery of FLICE (Muzio et al., 1996), a proapoptotic protease, which binds to FADD.



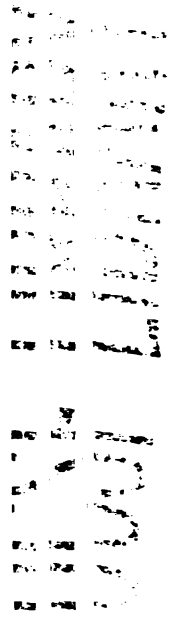
2-D PAGE

2-D PAGE separates proteins by both isoelectric point and molecular weight. In the past, 2-D PAGE relied on carrier ampholytes during isoelectric focusing (IEF) to establish and maintain the pH gradient (O'Farrell, 1975). There were many disadvantages with this method, the most common being reproducibility and protein load capacity. Additionally, variability in gels produced from different laboratories made the creation of worldwide reference gels difficult. The advent of immobilized pH gradients (IPG) gels, where the pH gradient is covalently linked with the acrylamide, has greatly

reduced this variability and allowed for a high degree of reproducibility (Bjellqvist et al., 1982; Görg et al., 1988; Righetti and Bossi, 1997). The use of immobilized pH gradients in the first dimension has also increased the amount of protein that can be loaded. Traditional IEF have a capacity of one milligram of protein, whereas immobilized pH gradients allow loading of up to five milligrams (Bjellqvist et al., 1993; Rabilloud et al., 1994). Because of reproducibility, ease of use, and unparalleled resolving power, the latter technique is now most commonly used for the separation of complex protein mixtures.

Many proteins, such as those that span the plasma membrane, are traditionally difficult to solubilize (Herbert, 1999; Molloy, 2000). High levels of sodium dodecyl sulfate (SDS) are commonly used to extract these proteins. However, charged species are usually minimized during IEF since separation is by charge. Immobilized pH gradients have even more stringent requirements due to their low tolerance of ionic detergents including SDS. The use of different chaotropic agents such as thiourea, surfactants e.g. (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and reducing agents including dithiothreitol (DTT) and tributyl phosphine, have greatly improved the efficiency of solubilization of hydrophobic proteins (Molloy, 2000). Also serial extractions with various lysis buffers increases the solubilization of additional proteins in lysates (Molloy et al., 1998). However, this method can lead to inconsistent recoveries after each step and introduces errors that preclude quantitative comparisons of protein levels in various conditions. For these reasons, we chose a single extraction with a lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS and 100 mM DTT.

After solubilization of proteins and separation by 2-D PAGE, proteins are visualized by staining the gels with various dyes. Commonly, Coomassie Brilliant Blue and silver stain are used for this purpose. Coomassie-based stains have the advantage of linearity over a wide range of protein concentration whereas silver stains allow for increased levels of sensitivity in detection. In this study, we used Fast-stain®, a Coomassie based dye, because the experiment we conducted which focused on examining the effects of oxygen required a wide range of detection rather than sensitivity. Ongoing improvements in fluorescent dyes such as the recently described Sypro Ruby allow for the detection of even lower levels of protein while maintaining wide ranges in linearity of detection (Celis and Gromov, 1999). In summary, continued developments in reagents for sample preparation and solubilization and stains for protein detection will increase the analytical powers of 2-D gels.



As methods for 2-D PAGE have improved, there has also been a coevolution of software for analysis of these gels. The new software packages allow a user to scan the gels and store the image. Protein expression levels can also be quantified. This facilitates searches for differences in protein patterns. As a result, these packages allow comparison and analysis of the vast amounts of information conveyed from these gels. In this study, we used the ImageMaster 2D software. Similar to other programs, ImageMaster detects, quantitates, and matches spots from multiple gels. In addition to these basic functions, ImageMaster has a querying function that allows the user to focus on a subset of gel spots. For example, one can identify and only focus on protein spots whose levels of expression are upregulated by a specified amount.

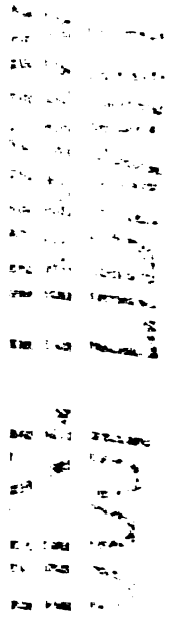
Mass Spectrometry (MS)

The development of two soft ionization techniques, matrix assisted laser desorption/ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn et al., 1989), in the late 1980s has helped to link the fields of MS and biology. Recent advances have made these techniques ideal for the rapid and accurate identification and characterization of proteins in peptide mixtures. For example, postsource decay (PSD) can be used to obtain partial peptide sequence (Spengler et al., 1992). Instruments which couple a continuous ionization source with a time-of-flight (TOF) analyzer, such as ESI-TOF mass spectrometers, help to circumvent limits imposed by quadrupole mass filters, which are commonly used with ESI sources, such as the tradeoff between sensitivity and resolution (Chernushevich et al., 1999; Verentchikov et al., 1994). Sample introduction methods have also improved. For example, nanospray (Wilm and Mann, 1996; Wilm et al., 1996) and microscale high pressure liquid chromatography (HPLC)-ESI sources (Costello, 1999; Davis and Lee, 1998; Davis et al., 1995; Shevchenko et al., 1997) facilitate delivery. Due to the advent of these improvements, current instruments allow the application of proteomics approaches to samples that contain relatively low levels of proteins.

MS has been widely used in proteomics and constitutes the core of most studies. It is compatible with not only 2D-PAGE, but also quantitative proteomics methods being developed which would remove the need for quantitation by gel electrophoresis such as isotope-coded affinity tags (Gygi et al., 1999). By using mass spectrometry, novel proteins such as FLICE (Muzio et al., 1996), Rabex-5 – a protein involved in membrane fusion (Horiuchi et al., 1997) – and Nem1p, which participates in the formation of the

spherical nucleus in yeast (Siniossoglou et al., 1998), have been identified. Post-translational modifications (Broekhuis et al., 2000; Kalo and Pasquale, 1999) and xenobiotic covalent adducts with proteins (Qiu et al., 1998) have also been identified.

The following chapters describe results that were obtained in three different projects that employed mass spectrometry and/or proteomics techniques. The major focus was the identification of oxygen-regulated placental proteins. Additionally, MALDI-TOF/PSD allowed the identification of two other proteins in different experimental systems.



Chapter 2. Functional Proteomics: Examining the Effects of Hypoxia on the Human First Trimester Cytotrophoblast Protein Repertoire.

Summary

The outcome of human pregnancy depends on the differentiation of cytotrophoblasts, specialized placental cells that physically connect the embryo/fetus to the mother. As cytotrophoblasts differentiate, they acquire tumor-like characteristics that enable them to invade the uterus. In a novel feedback loop, the increasingly higher levels of oxygen they encounter within the uterine wall influence their differention into vascular-like cells. Together, the invasive and cell surface properties of cytotrophoblasts enable them to form vascular connections with uterine blood vessels that divert maternal blood flow to the placenta, a critical hurdle in pregnancy. It is therefore important to understand, at a fundamental level, how cytotrophoblasts respond to changes in oxygen tension. Here we used a proteomics approach, 2-D PAGE combined with mass spectrometry, to characterize the protein repertoire of first trimester human cytotrophoblasts that were maintained under standard tissue culture conditions (20% O_2). 2-D PAGE showed a unique protein map as compared to placental fibroblasts and human JEG-3 choriocarcinoma cells. Mass spectrometry allowed the identification of 43 spots on the cytotrophoblast map. Enzymes involved in glycolysis and responses to oxidative stress, as well as the 14-3-3 signaling/adapter proteins, were particularly abundant. Hypoxia *in vitro* (2% O_2) produced discrete changes in the expression of a subset of proteins in all the aforementioned functional categories. Together, these data offer new information about the early gestation cytotrophoblast protein repertoire and the

generalized mechanisms the cells use to respond to changes in oxygen tension at the maternal-fetal interface.

Introduction

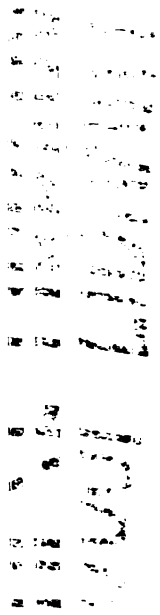
A great deal has been learned about normal cytotrophoblast differentiation by *in situ* immunolocalization of candidate molecules on tissue sections of the maternal-fetal interface (e.g., Figure 2B). Because isolated stem cells plated on extracellular matrix substrates recapitulate the differentiation process that leads to invasion *in vivo* (see Chapter 1 and diagram in Figure 4A and photomicrograph in Figure 4B), this culture model has been used to study the function of candidate regulatory molecules. Data generated by using a combination of *in situ* and *in vitro* approaches have led to a better understanding of placental development at a molecular level. As previously discussed in Chapter 1, cytotrophoblast differentiation is governed by an interesting set of transcription factors and their downstream targets. Among the transcription factors with known actions are both positive and negative regulators of the basic helix-loop-helix family (Cross, 1998; Janatpour et al., 2000) and the novel factor glial cells missing (Janatpour et al., 1999; Schreiber et al., 2000). Downstream targets include a number of stage-specific antigens, such as adhesion molecules, that allow cytotrophoblasts to mimic the surface of endothelial cells (Damsky and Fisher, 1998), and proteinases that likely play a role in invasion (Alexander et al., 1996; Bass et al., 1997; Librach et al., 1991). Among the most interesting antigens in this category are molecules that protect cytotrophoblasts within the uterine wall from the immune response typically elicited in

transplant situations by other hemiallogeneic cells. These include HLA-G, a major histocompatibility complex class Ib molecule (McMaster et al., 1995).

In comparison to published information about the properties of normal cytotrophoblasts, relatively little is known about the effects of pregnancy complications on these cells. With regard to shallow invasion in preeclampsia, cytotrophoblasts within the uterine wall fail to correctly modulate the expression of a number of stage-specific antigens, many of which are related to the acquisition of an adhesion molecule repertoire that is usually associated with vascular cells (Zhou et al., 1993; Zhou et al., 1997a). Interestingly, some aspects of the phenotype of cytotrophoblasts in preeclampsia can be replicated *in vitro* by culturing the cells under hypoxic conditions (Genbacev et al., 1996; Genbacev et al., 1997). Together, these observations suggest the hypothesis that abnormal differentiation and hypoxia are critical components of the pathway that leads to this syndrome. With regard to excessive invasion in choriocarcinoma, much of the existing information lies at the genetic level (Hui et al., 2000).

What approaches can be used to obtain new information about the pathways and the processes that are critical to both normal and abnormal placental development? The growing number of transgenic mice that have been generated to study the intraembryonic functions of particular molecules, but yielded placental phenotypes instead (Cross, 2000), illustrates the difficulties inherent in candidate, single molecule approaches. Conversely, gene array technologies often identify many more candidate molecules than it is feasible to study (Tanaka et al., 2000). Here we used a proteomics approach, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) coupled with mass spectrometry, to map cytotrophoblast protein expression at a global level in normal pregnancy. We also

examined the effects of hypoxia *in vitro* as a model of preeclampsia. The results showed that major cytotrophoblast proteins normally include an interesting set of molecules that are involved in carrying out glycolysis and handling oxidative stress. In addition, the 14-3-3 signaling proteins are abundant. Of note was the fact that the cytotrophoblast 2-D PAGE map, which was very different from that of placental fibroblasts, bore a greater resemblance to that of the JEG-3 human choriocarcinoma cell line. Hypoxia produced discrete changes in the cytotrophoblast protein repertoire and resulted in nuclear localization of 14-3-3-epsilon. Together, our results suggest that the cytotrophoblast protein repertoire is specially adapted to handle fluctuations in the cells' metabolic state and oxygen environment as they invade the uterus and form vascular connections with the maternal vessels.



MATERIALS AND METHODS

Materials. Matrigel and Dispase were from Becton-Dickinson (Bedford, MA). Nutridoma-HU was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Millicell-CM cell culture inserts (12-mm) were from Millipore Corporation (Bedford, MA). Bio-Rad Protein Assay reagent was from Bio-Rad (Hercules, CA). Immobiline Drystrips and Pharmalyte pH 3-10 were from Amersham Pharmacia Biotech (Piscataway, NJ). Fast-stain® was acquired from Zoion Research (Shrewsbury, MA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI). α-cyano-4-hydroxycinnamic acid was from Hewlett-Packard (Böblingen, Germany). Nitrocellulose membranes (0.45 μm) were obtained from Schleicher & Schuell (Keene, NH). A rabbit polyclonal antibody to 1-Cys peroxiredoxin and purified recombinant 1-Cys peroxiredoxin were the kind gift of Dr. Vladimir I. Novoselov (Russian Academy of Sciences, Pushchino, Russia) (Novoselov et al., 1999). A mouse monoclonal antibody to annexin II and lysates of MDCK cells were from Zymed Laboratories, Inc. (South San Francisco, CA). A rat monoclonal antibody to cytokeratin (Damsky et al., 1992) and a mouse monoclonal antibody to HLA-G (McMaster et al., 1995) were produced in this laboratory. Affinity-purified rabbit polyclonal antibodies to 14-3-3 epsilon and zeta were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-mouse IgG (heavy and light chains), horseradish peroxidase-conjugated donkey anti-rabbit IgG (heavy and light chains), biotin-conjugated donkey anti-rabbit IgG (heavy and light chains), ChromPure donkey IgG (whole molecule), and rhodamine-conjugated donkey anti-rat IgG (heavy and light chains) were from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Enhanced chemiluminescence

(ECL) kits were from Amersham Pharmacia Biotech. Avidin/biotin blocking kits, fluorescein-conjugated streptavidin, and Vectashield were from Vector Laboratories, Inc. (Burlingame, CA). All other reagents were acquired from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Placentas were obtained immediately after first trimester terminations (8-12 wk). Cytotrophoblasts were isolated from pools of multiple placentas by published methods (Librach et al., 1991). Fibroblast contamination, as measured by staining with an anti-vimentin antibody, was $\leq$ 5%. The resulting cells were plated on a Matrigel substrate (~100 μm thick) in serum-free medium (Dulbecco's modified Eagle's medium with 2% Nutridoma-HU, 1% glutamine, and 50 μg/ml gentamycin). The cultured cells were maintained as previously described under either standard tissue culture conditions (5% CO_2/95% air) or in hypoxia (2% O_2/5% CO_2/93% N_2) (Genbacev et al., 1996). After 48 h, cells were released from the Matrigel by treatment with Dispase and isolated by centrifugation (8 min, 400 x g) in a Sorvall RT 6000D centrifuge (Kendro Laboratory Products, Newtown, CT). The cell pellets were washed three times with Ca^{+2}- and Mg^{+2}-free phosphate-buffered saline (PBS-CMF) and frozen as a pellet prior to lysis.

Anchoring villi were prepared for culture as previously described (Genbacev et al., 1993; Genbacev et al., 1992). Briefly, small fragments of placental tissue from the maternal-fetal interface were teased apart until they had the characteristic tree-like appearance of chorionic villi as viewed in a stereo microscope. Anchoring villi were identified by the attached remnants of cell columns. Light microscopic examination of hematoxylin-stained sections of villus tissue preparations consistently showed the

presence of floating and anchoring villi and the absence of endometrial contamination. Anchoring villi (wet weight, 5-10 mg) were transferred to Matrigel-coated 12-mm Millicell-CM culture dish inserts (0.4 μm). The inserts were then placed into 24-well dishes and cultured up to 3 days in a mixture of Dulbecco's modified Eagle's medium/F-12 Ham's (1:1/v:v) culture medium supplemented with 0.1% of an antibiotic/antimycotic preparation (500 units penicillin, 1 mg streptomycin, 1.25 μg amphotericin B/ml; Sigma A-7295) and 2% Nutridoma-HU. Chorionic villus explants were cultured as previously described in either standard conditions (20% O_2) or hypoxia (2% O_2) (Genbacev et al., 1997).

Fibroblasts, isolated from first trimester placentas as previously described (Fisher et al., 1989), were used after the third passage to ensure that contaminating cells were no longer present. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% glutamine, and 50 μg/ml gentamycin. The JEG-3 choriocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Eagle's Minimal Essential Medium with Earle's salts, 1% L-glutamine and 10% fetal calf serum. When confluent, both fibroblasts and JEG-3 cells were released from the substrate with Dispase and isolated by centrifugation. The cell pellets were washed three times with PBS-CMF and frozen as a cell pellet prior to lysis.

2-D Polyacrylamide Gel Electrophoresis. Cells were lysed in protein extraction buffer (7 M urea, 2 M thiourea, 4% (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (CHAPS), 100 mM dithiothreitol (DTT) and 1% Pharmalyte pH 3-10)

and lysates were centrifuged at 356,000 x g for 10 min in a Beckman Optima TL Tabletop Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The supernatant was removed and the protein concentration of the extracts was determined by using the Bio-Rad Protein Assay reagent (Bradford, 1976). Immobiline DryStrips (180 mm, pH 3-10 L) were rehydrated overnight in 400 µl of lysate that contained 250 µg of protein. Isoelectric focusing was carried out up to a total of 70 kVh at 20°C on a Multiphor II system (Amersham Pharmacia Biotech). After IEF, the strips were incubated for 20 min in equilibration buffer (50 mM Tris pH 6.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% (w/v) DTT, and for an additional 20 min in the same buffer containing 4.5% (w/v) iodoacetamide. SDS-PAGE was performed on 10% gels run at 10,000 mW on the Investigator System (Genomic Solutions, Chelmsford, MA). Proteins were visualized using Fast-stain®, a modified Coomassie Blue stain with increased sensitivity. Digital images of the 2-D PAGE maps were acquired using a Sharp JX-330 Desktop Scanning Unit. The gels were analyzed with the ImageMaster 2D v 2.0 software from Amersham Pharmacia Biotech. Protein spot comparisons were done according to prescribed software instructions. Briefly, the computer program identified protein spots from the digitized images of the gels. In all cases spot identification was verified by visual inspection of the gels. Then we used the software program to compare spots from different gels. Background staining, defined as the average intensities of pixels bordering the spots, was subtracted from the gel spots.

In-Gel Trypsin Digestion of Proteins and Mass Spectrometry. After gels were destained in 10% acetic acid, protein spots were excised and macerated with a scalpel. In-gel

trypsin digests were performed as previously described (Matsui et al., 1997). Briefly, the gel pieces were transferred to a microfuge tube and washed with 50% acetonitrile/25 mM ammonium bicarbonate. Protein gel spots were digested overnight with 0.5 μg trypsin at 37°C, and tryptic peptides were eluted with 50% acetonitrile/5% trifluoroacetic acid in H_2O. After concentration under reduced pressure in a SpeedVac Concentrator (Savant, Holbrook, NY), portions (typically $1/20^{th}$ of the total volume) of the unseparated tryptic digests were co-crystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive Biosystems DE-STR MALDI-TOF mass spectrometer equipped with delayed extraction operated in the reflector mode. Spectra were internally calibrated using trypsin autoproteolysis peaks.

For samples that required peptide separation by HPLC, an Eldex Micropro pump was used. Chromatographic runs were performed on a Michrom Bioresources MagicMS C18 column (0.2 x 50 mm; 5 μm particle size; 200 Å pore size) using a flow rate of 1 μl/min. The column was equilibrated with 7% acetonitrile/0.1% trifluoroacetic acid in H_2O. Peptides were eluted isocratically for 10 min followed by a linear gradient (0.95%/min) to a final mobile phase composition of 63% acetonitrile/0.082% trifluoroacetic acid in H_2O. HPLC fractions of one-2 μl were spotted directly onto a MALDI target with 1.5 μl of α-cyano-4-hydroxycinnamic acid. Postsource decay (PSD) sequencing was done as previously described (Clauser et al., 1999). Database searches with spectral information were done via MS-Fit and MS-Tag programs, which are available on the internet (http://prospector.ucsf.edu) (Clauser et al., 1999).

Immunoblotting. The amount of protein in cell lysates prepared from cytotrophoblasts cultured as described above under standard and hypoxic conditions was estimated by densitometry. Briefly, samples were separated by 1-D PAGE on 10% SDS bis/acrylamide gels and bands were visualized by using Fast-stain®. Digital images of the gels were acquired with a Sharp JX-330 Desktop Scanning Unit, then the intensities of all the pixels in each lane were summed using ImageMaster 2D software. The background intensities were subtracted and the amount of protein loaded in each lane was estimated using these values.

Portions of the cell lysates that contained equal amounts of protein were subjected to 10% SDS-PAGE and then transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubating for 1 h at room temperature in PBS-CMF containing 0.1% Tween-20 (T-PBS) and 5% Carnation non-fat dry milk (blocking buffer). The following antibodies were diluted (v/v) in blocking buffer at the ratios indicated: anti-1-Cys peroxiredoxin (1:1000), anti-annexin II (1:1000), anti-14-3-3 epsilon (1:500), anti-14-3-3 zeta (1:500), and anti-HLA-G (1:200). The membranes were incubated for 2 h at room temperature with one of the diluted antibodies. After washing three times with T-PBS, each membrane was incubated for 1 h at room temperature with the appropriate species-specific secondary antibody. Horseradish peroxidase-conjugated donkey anti-mouse IgG (diluted 1:4000 in blocking buffer) was used to detect anti-annexin II and anti-HLA-G. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (diluted 1:4000 in blocking buffer) was used to detect anti-1-Cys peroxiredoxin and anti-14-3-3 epsilon and zeta. Membranes were washed three times in T-PBS and processed for chemiluminescence with ECL detection kits according to the manufacturer's instructions.

ImageMaster 2D software was used to measure band intensities for comparison purposes. Finally, HLA-G levels, which are not regulated by oxygen tension, were used as a second method for estimating the amount of protein that was transferred to each lane of the blot (Genbacev et al., 1997; Janatpour et al., 2000).

Immunohistochemistry. Villus explants, cultured as described above under either standard or hypoxic conditions, were fixed, embedded, and sectioned as previously reported (Genbacev et al., 1997). Double indirect immunofluorescence was performed as follows. Nonspecific reactivity was blocked by using an avidin/biotin blocking kit from Vector Laboratories according to the manufacturer's instructions. Tissue sections were incubated overnight at 4°C in antibodies specific to cytokeratin (diluted 1:50 [v/v] in PBS), 14-3-3 epsilon (diluted 1:100 [v/v] in PBS) or 14-3-3- zeta (diluted 1:100 [v/v] in PBS). After washing in PBS, sections were exposed to rhodamine-conjugated donkey anti-rat IgG (diluted 1:200 [v/v] in PBS-CMF) and biotin-conjugated donkey anti-mouse IgG (diluted 1:200 [v/v] in PBS-CMF) for 30 min at 37°C. Slides were washed and incubated with fluorescein-conjugated streptavidin (diluted 1:200 [v/v] in PBS-CMF) at 37°C for 15 min. After washing, sections were mounted with Vectashield. Antibody reactivity was visualized by dual channel fluorescence imaging. As a control, the expression of each antigen was evaluated separately on adjacent sections to rule out the possibility of overlap of the emission spectra. Additional controls included omission of either the primary or secondary antibodies.

Results

Overview of Experimental Strategy. The strategy that was used is diagrammed in Figure 5. Cell lysates were prepared from cultures of early gestation human cytotrophoblasts that were either maintained for 48 h under standard tissue culture conditions (20% O_2) that allow the cells to differentiate/invade normally, or subjected for the same length of time to hypoxia (2% O_2), conditions that replicate the shallow invasion that is the hallmark of preeclampsia (Genbacev et al., 1997). Lysates were also prepared from placental fibroblasts and the JEG-3 choriocarcinoma cell line. Cellular proteins were separated by 2-D PAGE (10% acrylamide) and the protein spots were visualized by using a modified Coomassie blue stain, which was subsequently removed during the washing procedure. The area of the gel that contained a protein spot was excised, macerated and subjected to an "in-gel" trypsin digest followed by final extraction of the proteolytic peptides. In some cases, the total extracted peptides from each digest was directly analyzed and in other instances the peptides were first separated by HPLC. MALDI-TOF/PSD generated peptide mass fingerprint and sequence data. This information was used in database searches to identify the protein.

2-D PAGE. We began by mapping the protein repertoire of cytotrophoblasts cultured under standard conditions. The results are shown in Figure 6. After staining, the number of spots was estimated by using ImageMaster 2D software; approximately 250 spots were resolved. Since the analysis focused on primary cells, we also investigated whether there were substantial individual variations in the protein components of cytotrophoblast lysates prepared from different placentas, a possible confounding factor

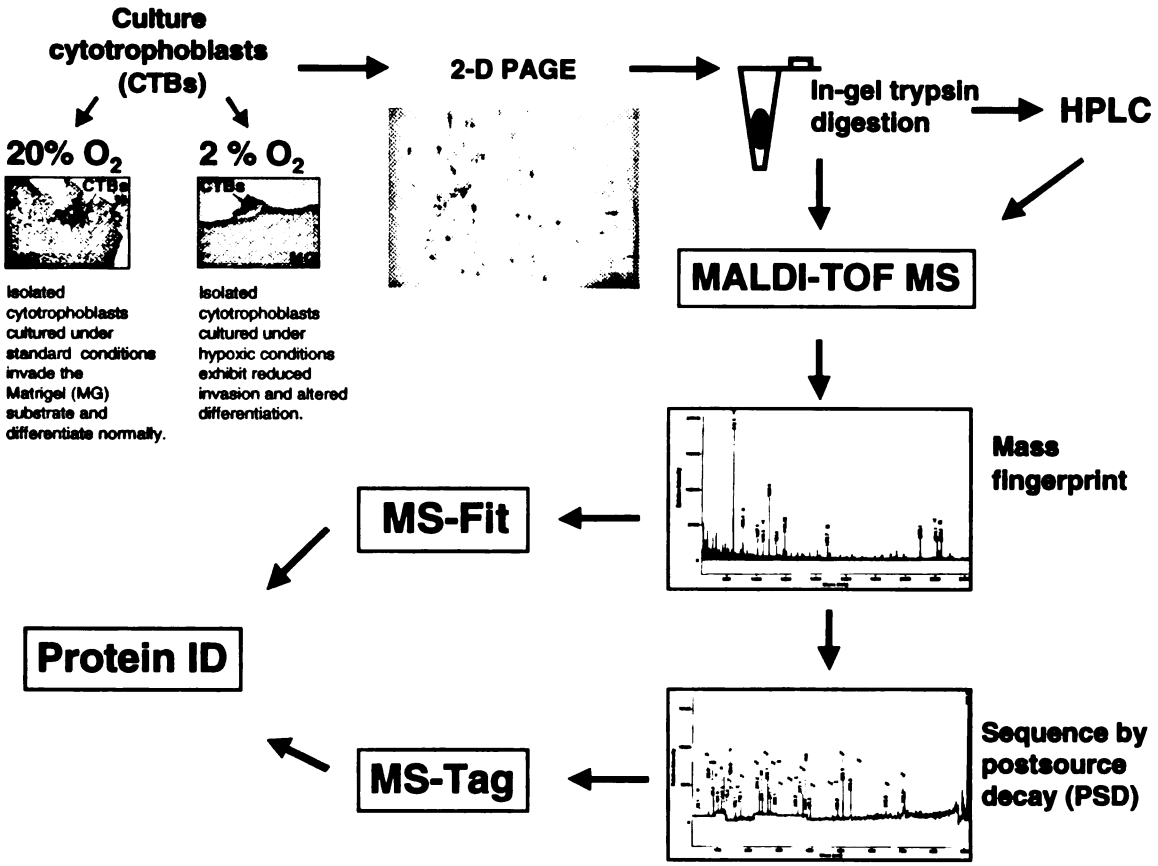


FIGURE 5. Diagram of the experimental strategy for proteomics-based study. Cytotrophoblasts were cultured under standard culture conditions (20% O_2), which trigger their exit from the cell cycle and differentiation along the invasive pathway, or hypoxic conditions (2% O_2) that cause them to proliferate rather than differentiate. As a result, invasion is also impaired and the cells sit atop the Matrigel (MG) substrate. Lysates were prepared from cells cultured under both conditions and subjected to 2-D PAGE. The proteins were visualized with Fast-stain®. Spots were excised for identification by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which yielded mass fingerprints. Fragment ions which provided sequence information were obtained by postsource decay (PSD) . The mass fingerprint and protein sequence information were used to search databases via the MS-Fit and MS-Tag programs, which allowed protein identification.

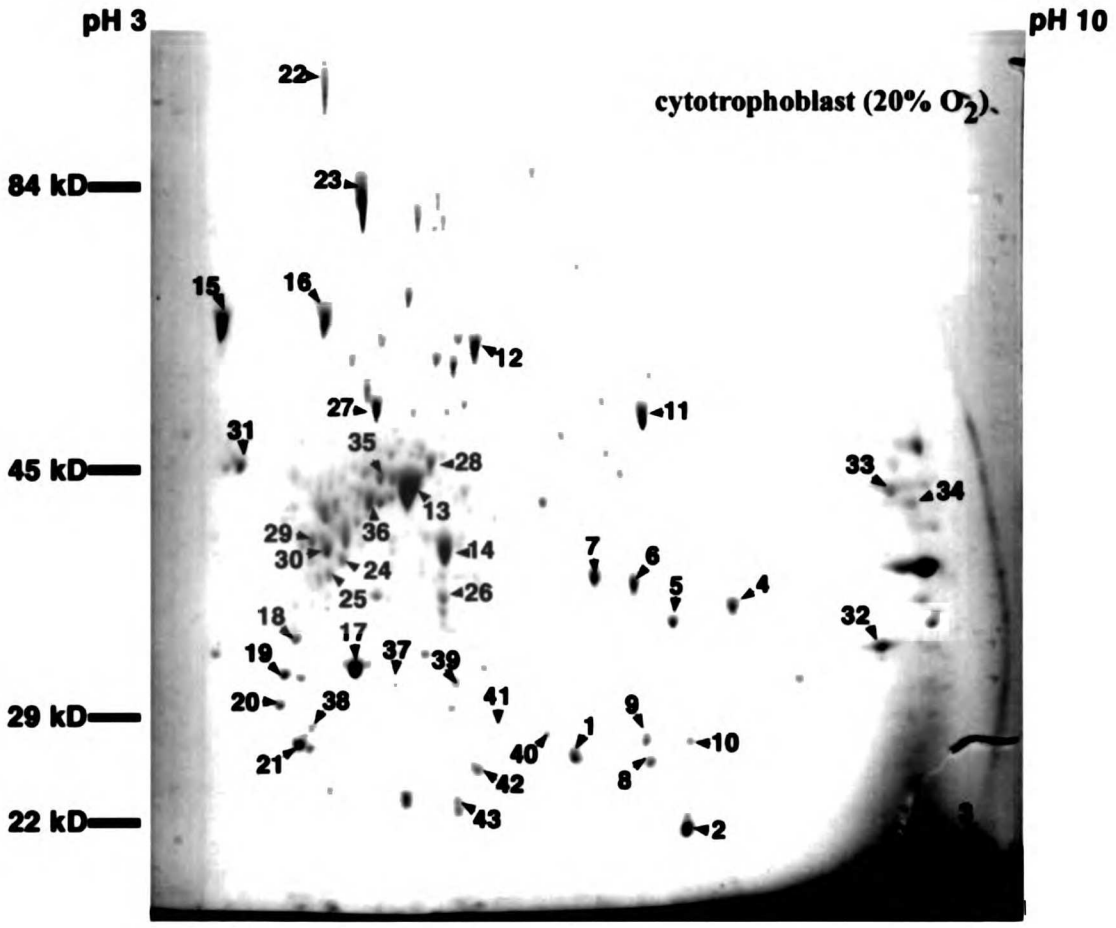


Figure 6. 2-D PAGE of lysates that were prepared from first trimester cytotrophoblasts cultured for 48 h in 20% O_2.

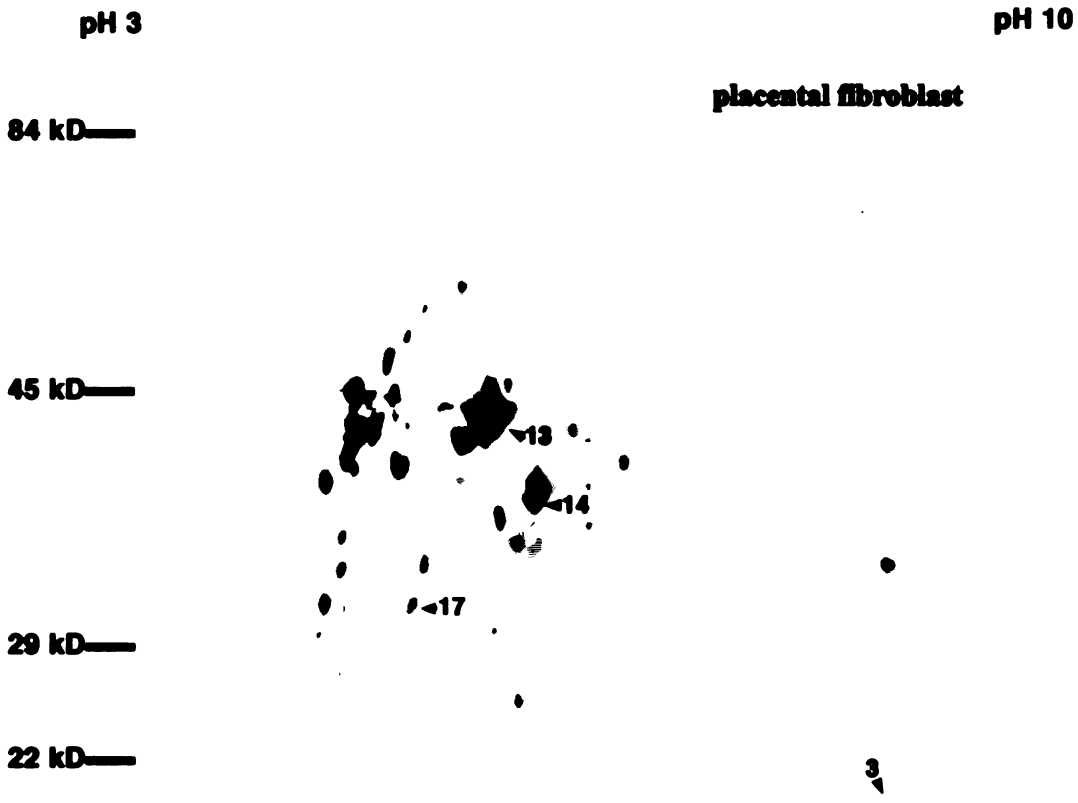


Figure 7. 2-D PAGE of lysates that were prepared from placental fibroblasts cultured for 48 h in 20% O_2. The pattern of cytotrophoblast spots bore little resemblance to that of placental fibroblasts, the other major cell type found in the placenta (see Figure 2A and B).

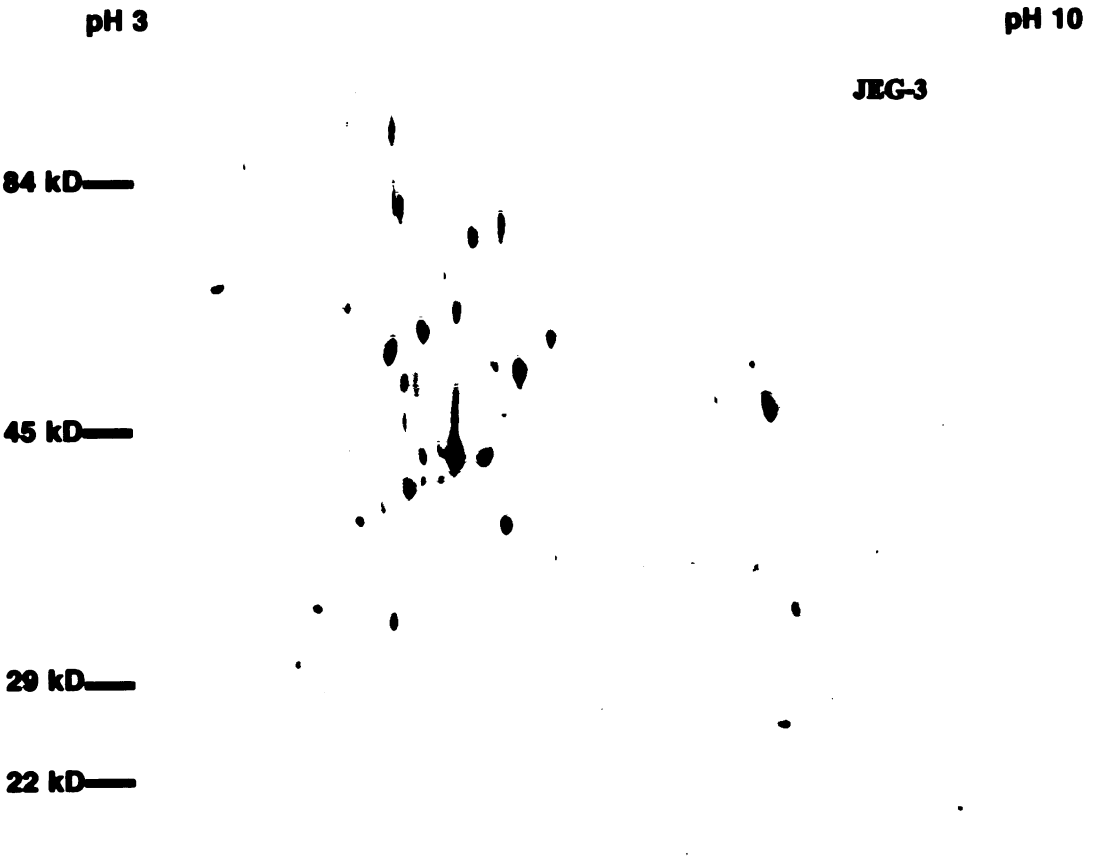


Figure 8. 2-D PAGE of lysates that were prepared from JEGs cultured for 48 h in 20% O_2.

for interpreting the results of subsequent experiments that investigated the effects of hypoxia on these cells. Accordingly, this experiment was repeated three additional times. No substantial variations in the repertoire or intensity of protein spots was detected, suggesting that the map shown in Figure 6 accurately reflected the cytotrophoblast protein repertoire within the confines of the analytical parameters of this experiment.

We also investigated whether the cytotrophoblast protein map was different from or similar to that of fibroblasts, the other major cell type found in the placenta. The fibroblasts that form the stromal cores of chorionic villi (see Chapter 1, photomicrograph in Figure 2B) yielded a very different protein map (Figure 7). The results showed that a few major proteins, such as spots 3, 13, 14 and 17, appeared at the same position on both maps, but most occupied unique locations (Figure 7), evidence that suggests the cytotrophoblast protein repertoire reflects the specialized biological functions these cells perform. Finally, we compared the cytotrophoblast 2-D PAGE map to that of the JEG-3 choriocarcinoma cell line. Although these cells have adapted to culture, their protein repertoire bore a greater resemblance to primary cytotrophoblasts than did that of placental fibroblasts (Figure 8).

MALDI-TOF MS/PSD. Our ultimate goal is to determine the identity of all the protein spots that are visible in Figure 6. To date we have used mass spectrometry approaches, primarily MALDI-TOF, to identify 43 different spots. Figures 9 and 10 shows examples of the spectra generated and the methods used to interpret them. The MALDI-TOF MS data from spots 1 and 4 are shown in Figure 9A and 10A, respectively. Peptides labeled "C" were present in a control blank gel plug and those labeled "T" are trypsin

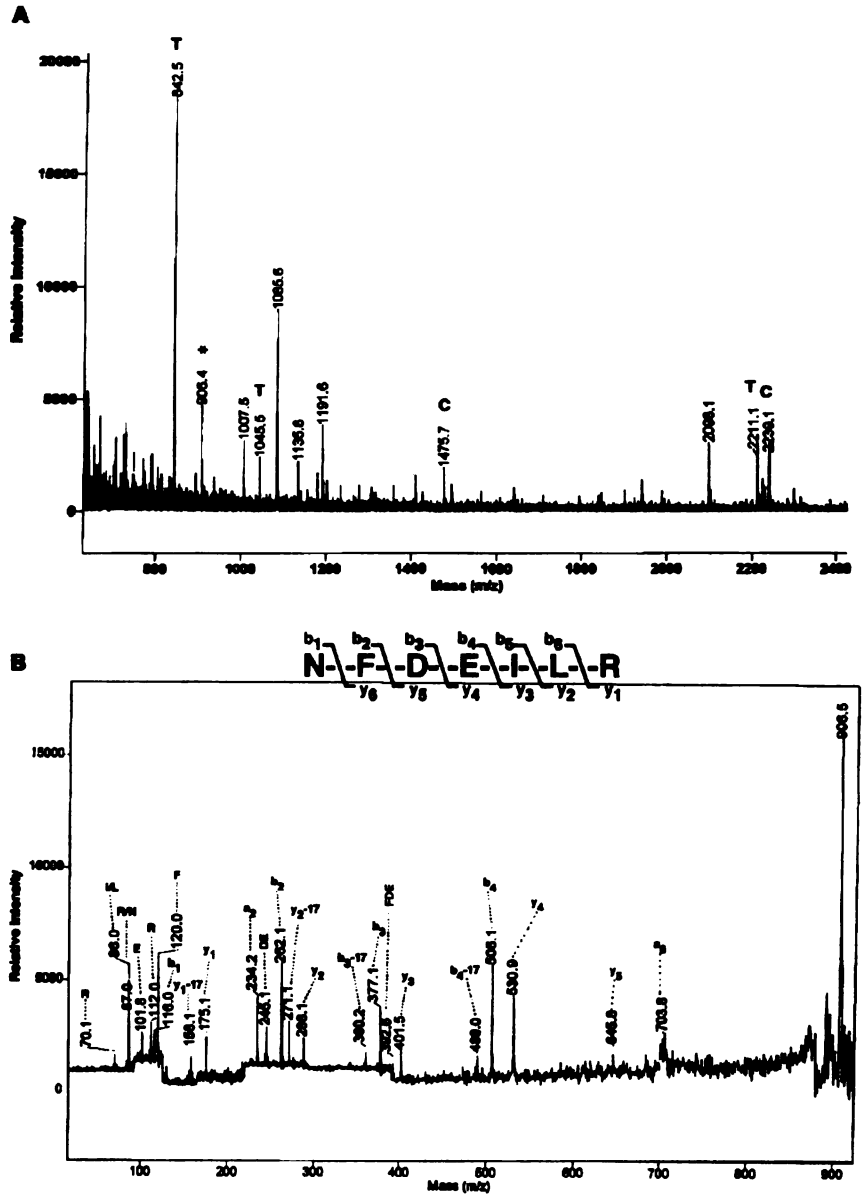


FIGURE 9: MALDI-TOF and PSD MS spectra of peptides obtained after in-gel trypsin digestion show that spot number 1 was 1-Cys peroxiredoxin. The spectra were acquired on a MALDI-TOF mass spectrometer operated in the reflectron mode. (A) MALDI-TOF MS spectra showing peptide mass fingerprints. Trypsin autoproteolysis products (peaks labeled "T") were used for internal calibration of spectra. Peaks labeled "C" were peptides detected in a control region of the polyacrylamide gel that did not stain for protein. (B) PSD spectra of ions marked with an asterisk in (A). Fragment types are indicated above peaks and the cleavage sites in the y- and b- series are labeled. The fragmentation pattern allowed assignment of the amino acid sequence illustrated above each spectrum. After database searches using information obtained from the analyses of several peptides (see Table 1), spot 1 was identified as 1-Cys peroxiredoxin.

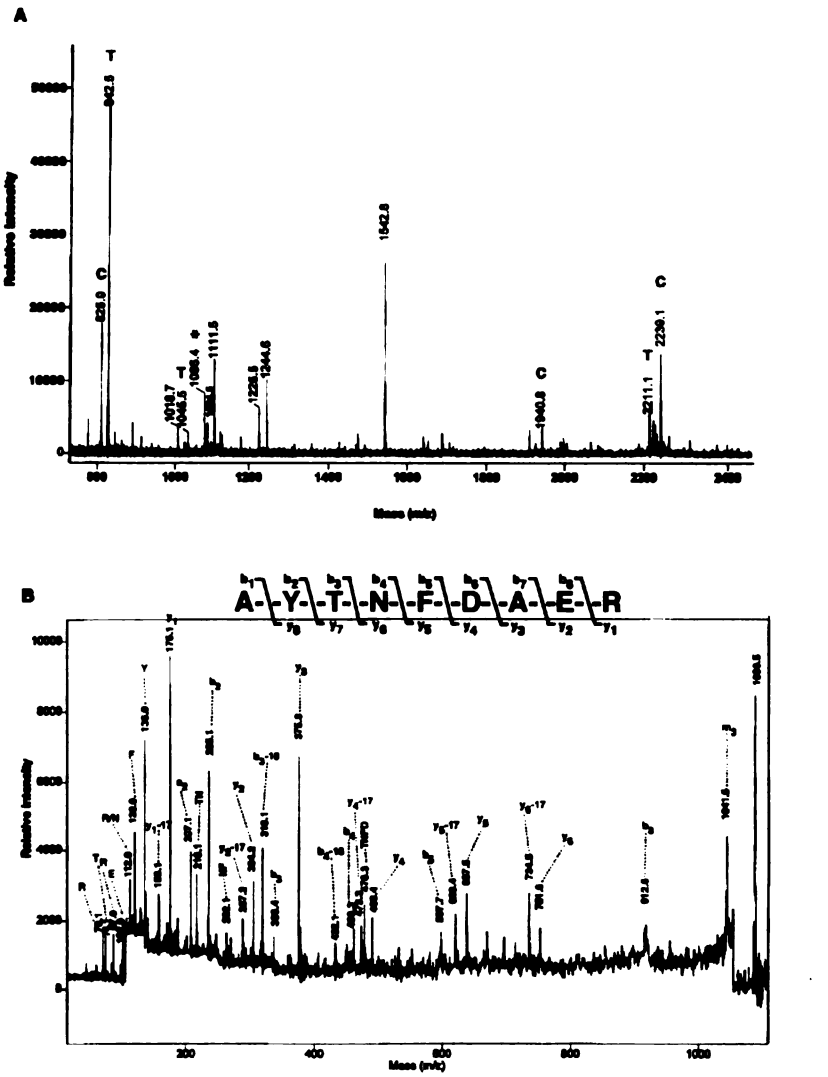


FIGURE 10: MALDI-TOF and PSD MS spectra of peptides obtained after in-gel trypsin spot number 4 was annexin II. (A) MALDI-TOF MS spectra showing peptide mass fingerprints. Trypsin autoproteolysis products (peaks labeled "T") were used for internal calibration of spectra. Peaks labeled "C" were peptides detected in a control region of the polyacrylamide gel that did not stain for protein. (B) PSD spectra of ions marked with an asterisk in (A). After database searches using information obtained from the analyses of several peptides (see Table 1), spot 4 was identified as annexin II.

autoproteolytic peptides. Unlabeled peaks were assumed to be from the protein in the gel spot of interest. Peptides, such as those designated by an asterisk in the MALDI spectra in Figures 9A and 10A, were subjected to PSD analysis. The resulting peptide fragmentation patterns (i.e., a-, b- and y-series ions), shown in Figure 9B and 10B, respectively, gave the amino acid sequence information shown. The masses and sequences of several peptides were used in database searches to identify the protein. For identification of gel spot 1, mass information from 6 peptides and the sequence information from a subset of 4 showed that this protein was the human 1-Cys peroxiredoxin. In the particular case of gel spot 4, mass information from 6 peptides and the sequence information from a subset of 2 identified this protein as annexin II.

The results of the entire analysis are summarized in Table 1 where spot numbers, marked on the gel in Figure 6, are correlated with protein identity. The peptide masses and sequences, obtained as described for spots 1 and 4, are also shown in the table. The majority of proteins could be organized into functional groupings. These included the antioxidants manganese superoxide dismutase (Mn SOD), 1-Cys peroxiredoxin and AOE 37-2. Several enzymes in the glycolytic pathway were also identified. With regard to protein chaperones, we detected HSP 27, HSP 70 and GRP-94 (the endoplasmic reticulum form of HSP 90), and molecules that mediate protein folding—protein disulfide isomerase (PDI), PDI-related proteins, ERp28, and cyclophilin. We also found four members of the annexin family of phospholipid-binding proteins. Calcium-binding proteins included calreticulin and calumenin. Finally, we found that three members of

Table 1. Summary of MS analyses of cytotrophoblast proteins identified from 2-D gels.

PROTEINS THAT FUNCTION AS ANTIOXIDANTS			
Spot #	**Protein (MW)**	**% coverage**	**MH$^+$ of tryptic peptides (Da)[a] (start-end position)**
2	Superoxide dismutase (Mn) (24,722.2)	30	815.3 (124-130), ***1004.5***[b] (195-202), ***1028.6***[b] (115-123), 1424.8 (76-89), 1738.8 (54-68), 1743.8 (203-216)
1	1-Cys peroxiredoxin (25,035.1)	29	***906.4***[b] (156-162), 1007.5 (133-141), ***1085.5***[b] (98-106), 1135.6 (133-142), ***1191.6***[b] (145-155), ***2098.1***[b] (2-22)
42	Antioxidant enzyme (AOE) 37-2 (30,540.1)	30	780.4 (166-173)[c], 797.5 (166-173), 819.4 (224-230), 1208.7[c] (213-223), 1212.6 (231-240), 1225.7 (213-223), 1464.8 (174-186), 1624.8 (187-200), 2185.9 (48-66), 2443.1 (46-66)
PROTEINS THAT FUNCTION IN GLYCOLYSIS			
Spot #	**Protein (MW)**	**% coverage**	**MH$^+$ of tryptic peptides (Da)[a] (start-end position)**
5	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (36,053.3)	27	***811.4***[b, d] (228-234), 909.5 (108-117), 1411.8 (201-215), 1544.8 (235-248), ***1613.9***[b] (67-80), 1763.8 (310-323), 1767.0 (201-219)

Table 1 (cont.)

PROTEINS THAT FUNCTION IN GLYCOLYSIS (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
8	Triose phosphate isomerase (TIM) (26,669.6)	27	1234.6 (195-206), 1414.8 (20-33), 1458.7 (101-113), 1466.7 (176-188), 1542.9 (19-33), 1637.8[c] (70-85)
9	Phosphoglycerate mutase (28,804.1)	15	1059.6 (91-100), 1150.7 (181-191), 2131.1[d] (223-240)
11	Enolase (47,169.2)	21	806.5 (407-412), 1143.6 (184-193), 1406.7 (16-28), 1425.7 (270-281), 1556.8[d] (240-253), ***1804.9***[b] (33-50), 2033.1 (307-326)
33	Phosphoglycerate kinase (43,967.2)	18	1101.5 (31-39), ***1634.8***[b] (157-171), ***1769.0***[b] (201-216), 2023.0 (280-297)
34	Aldolase (39,289.0)	33	937. 5 (322-329), ***940.5***[b] (14-21), ***1027.5***[b, c] (60-68), ***1044.6***[b] (60-68), 1342.7 (87-98), 1434.7 (1-12), 1646.8 (42-55), 1691.8[d] (243-257), 2123.1[d] (153-172), 2272.1 (111-133)
PROTEINS THAT ACT AS CHAPERONES/MEDIATORS OF PROTEIN FOLDING			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
40	Heat shock protein 27 (HSP 27) (22,782.6)	14	***1163.6***[b] (28-37), ***1906.0***[b] (172-188)

Table 1 (cont.)

PROTEINS THAT ACT AS CHAPERONES/MEDIATORS OF PROTEIN FOLDING (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH^+ of tryptic peptides (Da)[a] (start-end position)**
23	BiP (HSP 70 FAMILY) (72,160.0)	45	919.5[d] (262-268), 986.5 (533-540), 997.5 (298-306), 1074.5 (524-532), 1191.6 (465-474), 1228.6 (50-60), 1233.6[d] (186-197), 1316.7 (563-573), 1329.6[d] (327-336), 1397.8 (622-633), 1430.7 (102-113), 1460.8 (354-367), 1528.7[d] (325-336), 1566.8 (61-74), 1588.8 (353-367), 1604.9 (124-138), 1659.9 (198-213), 1677.8 (82-96), 1816.0 (198-214), ***1888.0***[b] (165-181), ***1934.0***[b] (475-492), 1974.9 (602-617), 2165.0 (289-306), 2176.0 (633-653)
22	Glucose regulated protein-94 (GRP-94) (HSP 90 family) (92,469.3)	23	1015.5 (396-404), 1047.5[d] (538-547), 1081.5 (76-84), 1139.6 (494-503), 1150.5 (548-557), ***1187.7***[c] (385-395), 1278.6 (547-557), 1485.8 (742-753), 1515.7 (475-486), 1525.7 (253-265), 1529.8[d] (143-156), 1627.7[d] (416-428), ***1785.9***[b] (52-67), 2046.0 (117-135), 2260.0 (512-530)
12	Protein disulfide isomerase (PDI) (56,679.6)	31	877.5 (297-304), 1191.6 (63-73), 1236.5 (108-119), 1359.7 (352-362), 1370.7 (472-482), 1579.8 (483-496), 1619.8 (259-271), 1680.8[d] (434-448), 1832.9 (380-395), 2348.1 (153-173), 2575.3 (306-329)
16	Prolyl 4-hydroxylase beta-subunit/ PDI precursor (57,116.6)	33	***910.4***[b] (445-452), 966.6 (301-308), 991.5 (223-230), 1066.5 (453-461), 1081.7 (255-263), 1158.6 (32-42), 1202.6 (121-130), 1213.5 (376-385), 1222.6[d] (317-326), 1424.8 (196-207), 1451.7 (327-338), 1729.9 (410-424), ***1780.8***[b] (82-97), 1833.9 (286-300), 1965.0 (231-247)

Table 1 (cont.)

PROTEINS THAT ACT AS CHAPERONES/MEDIATORS OF PROTEIN FOLDING (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
27	Protein disulfide isomerase related protein-5 (46,199.1)	38	708.3 (368-373), 759.3 (391-396), 1078.6 (295-303), 1151.5 (227-237), 1191.6 (90-99), 1386.8 (100-113), 1396.6 (142-153), 1483.7 (355-367), 1514.7 (176-189), 1524.7 (141-153), 1527.8 (198-212), 1615.8 (374-390), 1825.9 (67-83), 2057.0 (1-18)
3	Cyclophilin (22,611.3)	38	812.4 (37-42), 880.5 (51-58), 896.4 (114-120), 1047.6[d] (163-171), 1244.7 (196-206), 1302.6[d] (90-100), 1364.7 (63-75), 1457.7 (137-149)
41	ERp 28 (28,993.6)	26	937.5 (198-204), 1134.7 (244-253), 1247.6 (60-69), ***1320.7***[b] (113-122), 1608.8 (123-137), 1676.9 (110-122), ***1724.8***[b, d] (209-223)
PROTEINS THAT BIND PHOSPHOLIPIDS			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
7	Annexin I (38,714.5)	34	908.4 (215-212), 1213.5 (167-177), 1262.6 (114-124), 1387.8 (59-71), 1550.8 (215-228), 1606.0 (99-113), 1702.9 (129-144), 1739.7 (189-204), 1905.0 (82-98)
4	Annexin II (38,604.2)	16	***1086.4***[b] (29-37), 1094.5[c] (69-77), 1111.5 (69-77), 1225.5 (158-168), 1244.6 (136-145), ***1542.9***[b] (50-63)
32	Annexin II (38,604.2)	34	1051.5[d] (213-220), 1094.5[c] (69-77), 1111.6 (69-77), 1244.6 (136-145), 1421.7 (314-324), 1476.7 (234-245), 1542.8 (50-63), 1651.0 (89-104), 1908.9 (180-196), 2065.0 (179-196)

Table 1 (cont.)

PROTEINS THAT BIND PHOSPHOLIPIDS (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
39	Annexin IV (35,882.9)	38	1091.5[d] (276-284), 1134.5 (142-150), 1279.7 (193-202), 1366.6[c] (173-184), ***1427.8***[b, d] (144-155), 1477.1[c] (173-185), ***1692.9***[b] (29-44), 1941.0[d] (254-270), ***2366.2***[b, d] (1-24)
17	Annexin V (35,804.8)	51	954.5 (193-200), 1001.6 (108-116), 1014.5 (89-96), 1106.6 (276-284), 1143.7[d] (79-88), 1155.6 (260-270), 1172.7[d] (151-160), 1290.6[d] (290-300), ***1340.6***[b] (6-17), 1418.7[d] (289-300), 1446.8 (63-75), 1613.9 (227-241), 1704.9 (29-44), 1749.9[d] (245-259), 1818.9[d] (212-226)
PROTEINS THAT BIND CALCIUM			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
15	Calreticulin (48,141.8)	47	772.4 (273-278), 975.5 (65-73), 992.5 (279-286), 1019.6 (144-151), 1047.5 (359-366), 1147.7 (143-151), 1219.7 (88-98), ***1410.6***[b] (25-36), 1490.7 (99-111), 1607.8 (74-87), 1800.8 (208-222)
31	Calumenin (37,072.9)	24	954.5 (235-241), 1109.5 (104-111), 1887.9 (256-271), 1987.9 (293-311), 2479.1 (38-59), 2701.3 (288-311)
PROTEINS INVOLVED IN SIGNALING			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
20	14-3-3 epsilon form (29,174.1)	33	***907.6***[b] (24-31), ***1205.7***[b, d] (197-206), 1256.6 (112-122), 1463.7[d] (11-23), 1835.9[d] (135-151), ***2088.0***[b] (178-196)

Table 1 (cont.)

PROTEINS INVOLVED IN SIGNALING (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
21	14-3-3 zeta (27,745.3)	44	948.4[d] (121-127), 1017.6 (84-91), 1124.6[d] (159-167), 1205.7[d] (213-222), 1252.6[d] (158-167), 1279.7 (128-139), 1304.7 (104-115), ***1548.7***[b] (28-41), 2041.0 (140-157), 2132.0 (194-212)
38	14-3-3 gamma (28,374.6)	10	***1205.7***[b, d] (216-225), ***1644.0***[b] (29-42)
PROTEINS THAT FUNCTION IN CELL MOTILITY OR STRUCTURE			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
18	Tropomyosin (32,876.1)	29	915.5 (192-198), 988.5 (183-191), 1073.6 (13-21), 1186.7 (169-178), 1210.6 (218-226), 1243.7 (92-101), 1314.8 (168-178), 1475.8[d] (141-152), 1597.8 (52-65), 1648.8[d] (137-149)
19	Tropomyosin (28,522.0)	14	1170.7 (133-142), 1243.8 (55-64), 1298.8 (132-142), 1399.8 (55-65), 1614.8 (14-27), 1742.9 (13-27)
13	Actin (41,813.1)	14	795.5 (329-335), 800.5 (62-68), ***1132.5***[b] (197-206), 1499.7[c] (360-372), 1516.7 (360-372), ***1790.9***[b] (313-328)
14	Actin (41,813.1)	14	1132.5 (197-206), 1516.7 (360-372), 1639.8[d] (178-191)), 1790.9 (313-328)
26	Actin (41,813.1)	18	1132.5 (197-206), 1499.7[c] (360-372), 1515.7 (85-95), 1516.7 (360-372), 1790.9 (313-328), 1954.1 (96-113)

Table 1 (cont.)

PROTEINS THAT FUNCTION IN CELL MOTILITY OR STRUCTURE (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH^+ of tryptic peptides (Da)[a] (start-end position)**
24	Cytokeratin 8 (53,562.3)	30	1079.5 (226-233), 1129.6 (342-352), 1137.6 (286-295), 1277.7 (382-392), 1344.7 (329-341), 1368.7[d] (187-197), 1419.7 (214-225), 1808.9[d] (161-176), 1879.8[d] (134-148), 2125.0[d] (234-252)
25	Cytokeratin 8 (53,562.3)	15	1079.5 (226-233), 1129.6 (342-352), 1277.7 (382-392), 1344.7 (329-341), 1419.7 (214-225)
28	Cytokeratin 18 (47,334.2)	38	758.4 (132-137), 807.4 (159-165), 837.4 (91-97), 889.5 (318-325), 965.5 (254-261), 975.5 (7-14), 982.4 (125-131), 1041.6 (150-158), 1065.6 (373-381), 1109.5[d] (82-90), 1250.6[c] (176-186), 1267.6 (176-186), 1292.7 (371-381), 1319.7 (138-149), 1402.7[c] (359-370), 1419.7 (359-370), 1522.7[d] (302-314), 1884.0 (223-241), 2193.2[d] (197-214)
29	Cytokeratin 19 (44,106.2)	54	948.5[c] (359-365), 965.4 (359-365), 993.5 (169-176), 1008.5 (112-118), 1029.6 (189-197), 1041.6 (151-159), 1073.6 (141-150), 1082.5 (266-274), 1120.5[d] (82-90), 1122.6 (373-381), 1220.6[d] (177-187), 1222.6 (167-176), 1227.6 (217-226), 1365.7 (371-381), 1370.6[d] (254-264), 1389.7 (318-330), 1418.7 (114-125), 1501.7[d] (281-293), 1674.8 (126-138), 1904.9 (382-398), 2014.0 (227-247), 2139.1[d] (198-215), 2407.3 (331-353)

Table 1 (cont.)

PROTEINS THAT FUNCTION IN CELL MOTILITY OR STRUCTURE (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
30	Cytokeratin 8 (53,562.3)	43	906.5 (111-117), 995.5[d] (305-312), 1000.6 (317-325), 1062.5[c] (226-233), 1066.5 (179-186), 1079.5 (265-273), 1129.6 (342-352), 1137.6 (286-295), 1169.6 (373-381), 1185.6[d] (276-285), 1277.7 (382-392), 1336.7[d] (253-264), 1344.7 (329-341), 1368.7[d] (187-197), 1419.7 (214-225), 1808.8[d] (161-176), 1879.8[d] (134-148), 2125.1[d] (234-252)
35	Cytokeratin 8 (53,562.3)	23	1062.5[c] (226-233), 1066.5 (179-186), 1079.5 (226-233), 1129.6 (342-352), 1277.7 (382-392), 1344.7 (329-341), 1368.7[d] (187-197), 1419.7 (214-225), 2125.0[d] (234-252)
36	Cytokeratin 19 (44,106.2)	32	850.5 (25-32), 948.4[c] (359-365), 965.4 (359-365), 993.5 (169-176), 1029.6 (189-197), 1041.6 (151-159), 1222.6 (167-176), 1227.6 (217-226), 1365.7 (371-381), 1370.6[d] (254-264), 1389.7 (318-330), 1501.7 (281-293), 1537.7[c] (8-24), 1554.8 (8-24), 1674.8 (126-138)
OTHERS			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
6	Aldose reductase (35,853.6)	19	694.3[d] (252-256), 892.5 (34-41), 901.6 (244-251), 1083.4 (309-316), ***1120.6***[b] (70-78), 2452.3[d] (156-177)
10	Carbonic anhydrase (29,218.1)	13	***935.5***[b] (81-89), 1169.5 (172-181), 1669.0 (133-148)

Table 1 (cont.)

OTHERS (cont.)			
Spot #	**Protein (MW)**	**% coverage**	**MH⁺ of tryptic peptides (Da)[a] (start-end position)**
37	Nuclear chloride ion channel (26,923.4)	15	957.5 (209-216), ***1037.5***[b] (196-204), 1458.7 (96-113)
43	Glutathione-s-transferase (23,224.8)	31	980.6 (4-11), ***1337.7***[b] (1-11), 1883.9 (55-70), 2126.2 (121-140), 2132.9 (82-100)

[a] The mass accuracy of tryptic peptides were within ± 0.05 Da.

[b] PSD was done on bold italicized peptides to obtain sequence information.

[c] Peptide contains an N-terminal pyro-glutamic acid.

[d] Peptide contains an oxidized methionine

the 14-3-3 family of adapter/signaling molecules were among the prominent Coomassie blue-stained spots.

Functional Proteomics: the Effects of Hypoxia on the Cytotrophoblast Protein Repertoire. A number of pregnancy complications, including preeclampsia, are thought to be related either directly or indirectly to reduced blood flow at the maternal-fetal interface (see Chapter 1, Figure 3), and consequently to placental hypoxia. Therefore, we were interested in the effects of hypoxia *in vitro* on cytotrophoblast protein expression as monitored by 2-D PAGE. Visual comparison of the maps of cytotrophoblasts maintained under standard and hypoxic conditions, Figures 11A and 11B respectively, showed that the intensity of relatively few protein spots changed when the oxygen tension was reduced to 2%. This finding is in agreement with our previous work that suggests these cells are unusually resistant to the effects of hypoxia, which in fact causes them to proliferate (Genbacev et al., 1996; Genbacev et al., 1997). We used the Pharmacia ImageMaster 2D system to compare the intensities of protein spots whose abundance appeared to be regulated by oxygen tension. This analysis showed that only six spots changed in abundance by > twofold. Levels of four proteins increased as indicated: triosephosphate isomerase (twofold), phosphoglycerate mutase (twofold), annexin II (twofold) and tropomyosin (twofold). In contrast, levels of two antioxidants, 1-Cys peroxiredoxin (threefold) and Mn superoxide dismutase (twofold), decreased. Together, these results suggest that hypoxia exerts discrete effects on the cytotrophoblast protein repertoire; the abundance of most proteins on the 2-D PAGE map did not change.

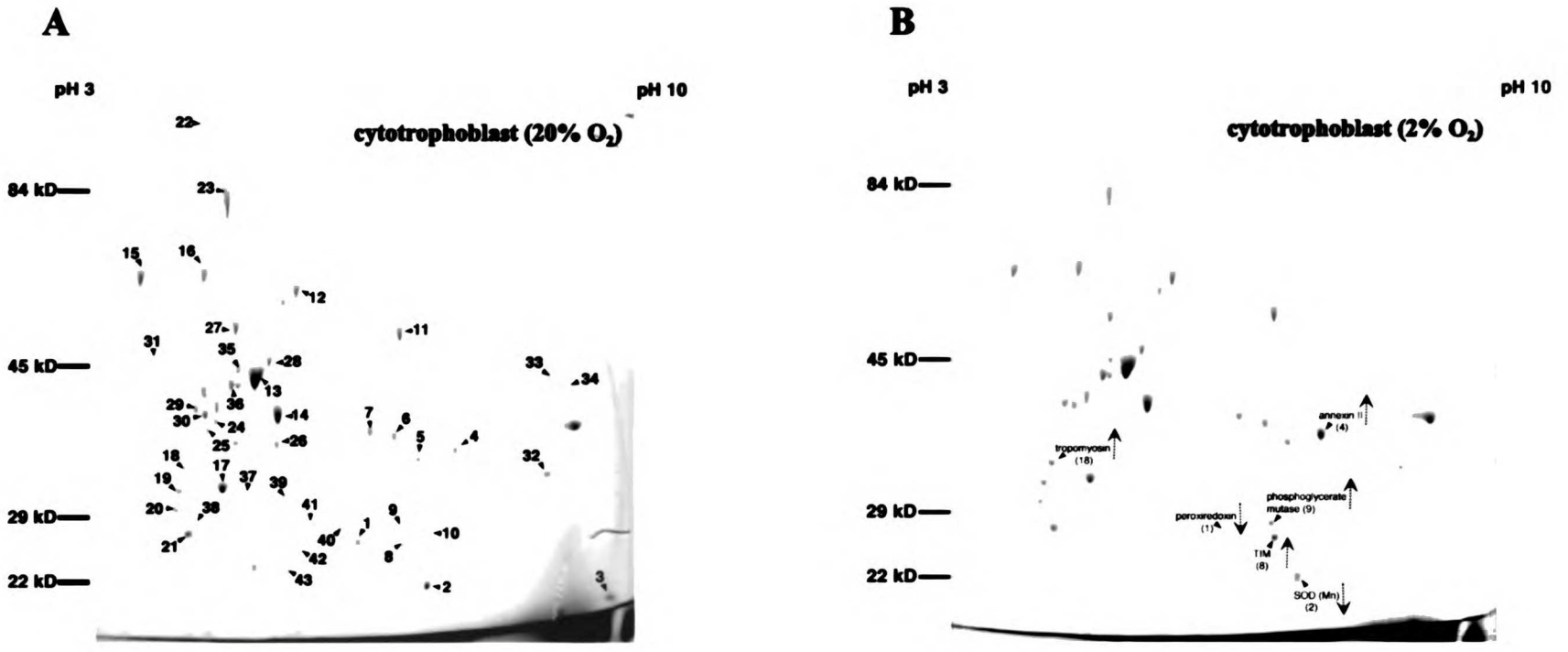


FIGURE 11: 2-D PAGE allowed identification of a subset of first trimester cytotrophoblast proteins that were oxygen regulated. (A and B) 2-D PAGE of lysates that were prepared from cytotrophoblasts cultured for 48 h in 20% or 2% O_2, respectively. Visual inspection, verified by analyses using Image Master 2D software (see Materials and Methods), suggested that the cytotrophoblast protein repertoire is largely stable in hypoxia; the abundance of six spots changed. Dashed arrows pointing upward indicate increased protein levels in hypoxia. Dashed arrows pointing downward indicate a decrease in protein levels in hypoxia.

Immunoblot Analyses of Cytotrophoblast Expression of 1-Cys Peroxiredoxin, Annexin II, 14-3-3 Epsilon, and 14-3-3 Zeta. We performed immunoblot analyses to confirm the identity and oxygen regulation (or lack thereof) of four cytotrophoblast proteins identified in the MALDI-TOF MS/PSD analyses. We analyzed the expression of one protein that was downregulated in hypoxia (1-Cys peroxiredoxin), one protein that was upregulated in hypoxia (annexin II) and, as controls, two members of the 14-3-3 family whose levels were not influenced by oxygen tension. Figure 12A shows the results of immunoblotting with anti-1-Cys peroxiredoxin. A single band of the expected size (26 kD) was detected in the lane that contained the recombinant protein. In four separate experiments, hypoxia induced a two- to eightfold reduction in cytotrophoblast levels of 1-Cys peroxiredoxin. This was in contrast to the results of immunoblotting with anti-HLA-G, which was used here and subsequently to compare within individual experiments the protein load of lysates prepared from control (20% O_2) and experimental cytotrophoblasts (2% O_2).

Figure 12B shows the results of immunoblotting with anti-annexin II. The antibody detected a single band of 38 kD in a MDCK cell lysate that served as a positive control. Likewise, cytotrophoblast lysates prepared from cells that were not treated with Dispase also contained a single band (data not shown). In contrast, most of the lysates prepared from Dispase-treated cells contained multiple immunoreactive bands, likely due to proteolysis of the antigen. Therefore, quantitation was accomplished by summing the signals from all the bands. These results showed that in three of five experiments annexin II levels rose between two- and fivefold in hypoxia, suggesting that this response

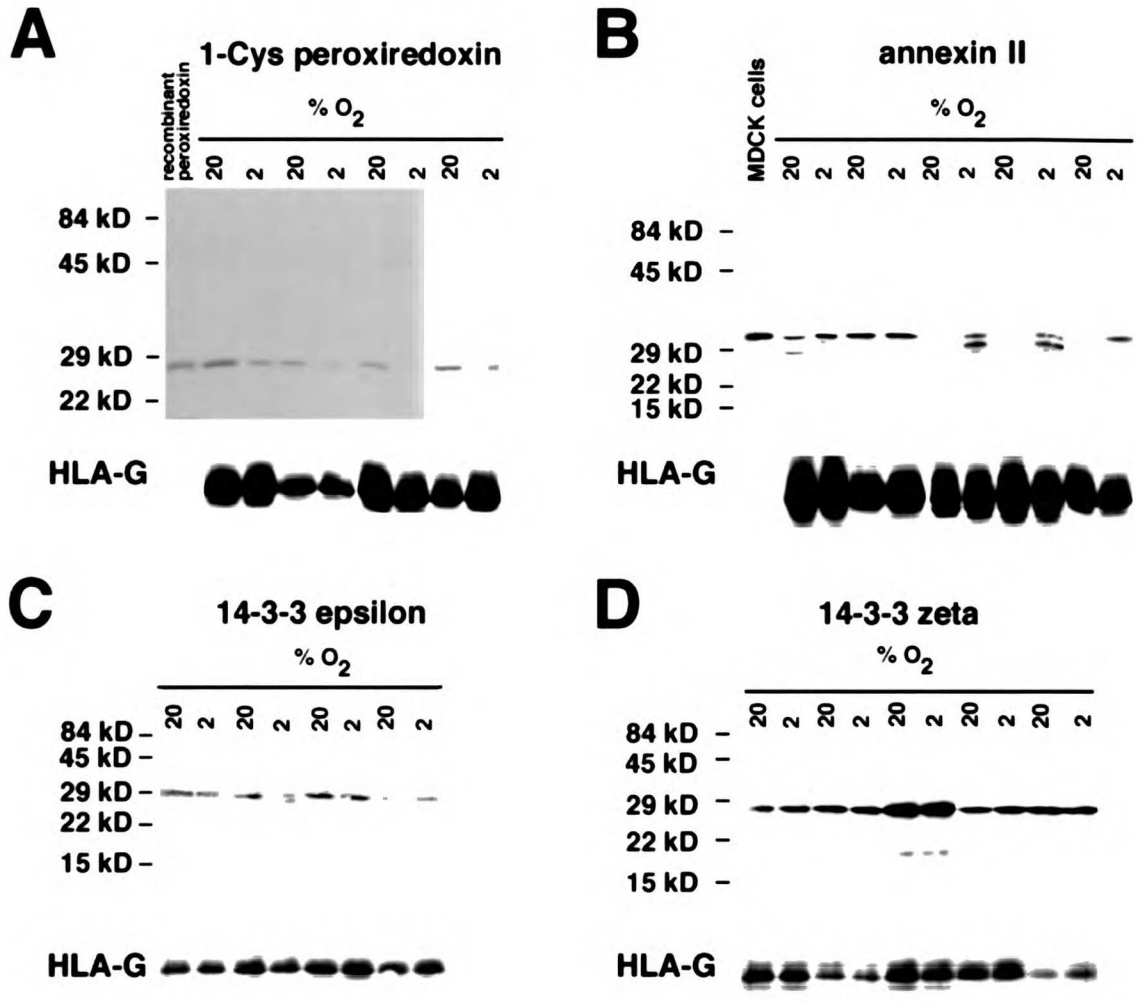


FIGURE 12: Immunoblot analyses confirmed that in cytotrophoblasts the levels of 1-Cys peroxiredoxin and annexin II, but not 14-3-3 epsilon and 14-3-3 zeta, were regulated by oxygen tension. (A) Immunoblot analyses confirmed that in four different cell preparations the levels of 1-Cys peroxiredoxin decreased in hypoxia. (B) In contrast, three of the five samples analyzed showed that annexin II levels increased in hypoxia, suggesting greater individual variations in the response. In this case, many of the cytotrophoblast samples contained multiple bands, not present in the standard MDCK lysates, that were attributable to Dispase treatment during harvesting of the cultured cells. Conclusions were based on summing the signals from all the bands. (C and D) As expected from the 2-D PAGE analyses (see Figure 3A and B), expression of neither 14-3-3 epsilon nor 14-3-3 zeta was regulated by oxygen tension. In all cases the blots were stripped and reprobed with an antibody against HLA-G, the cytotrophoblast class I molecule whose abundance does not change in hypoxia. The results, which are shown below each immunoblot, allowed comparison of protein loads within individual experiments (e.g., 20% vs. 2% O_2).

was somewhat less consistently observed in individual cytotrophoblast preparations than the downregulation of 1-Cys peroxiredoxin expression.

Next, we performed immunoblot analyses of two members of the 14-3-3 family. Anti-14-3-3 epsilon reacted with a single band of the expected size (29 kD) in all the cytotrophoblast lysates except one sample that contained a doublet (Figure 12C). The levels of this antigen did not change in hypoxia. Likewise, anti-14-3-3 zeta primarily reacted with a protein of the expected size (28 kD), and the levels were not affected by a reduction in oxygen tension (Figure 12D). Taken together, the results of the immunoblot analyses confirmed those obtained using the 2-D PAGE/MS approach.

Hypoxia Alters the Subcellular Localization of 14-3-3 Epsilon, but Not 14-3-3 Zeta. Finally, we investigated whether the subcellular localization, rather than the abundance, of the 14-3-3 signaling and adapter proteins changes in hypoxia. Immunolocalization experiments were performed on sections cut from villus explants cultured on Matrigel plugs in either a 20% or a 2% O_2 atmosphere (see Materials and Methods). The results are shown in Figure 13. The sections were stained with anti-cytokeratin to confirm that the cells were cytotrophoblasts (CK, Figure 12A and C). In 20% O_2 staining for 14-3-3 epsilon was primarily detected in association with the plasma membrane region of the cells (Figure 13B, denoted by arrow). In 2% O_2 many of the cytotrophoblasts showed nuclear staining (Figure 13D, denoted by arrowhead) in addition to plasma membrane antibody reactivity (Figure 13D, denoted by arrow). In contrast, the staining pattern for 14-3-3 zeta, which was also plasma membrane associated, was the same whether the cells

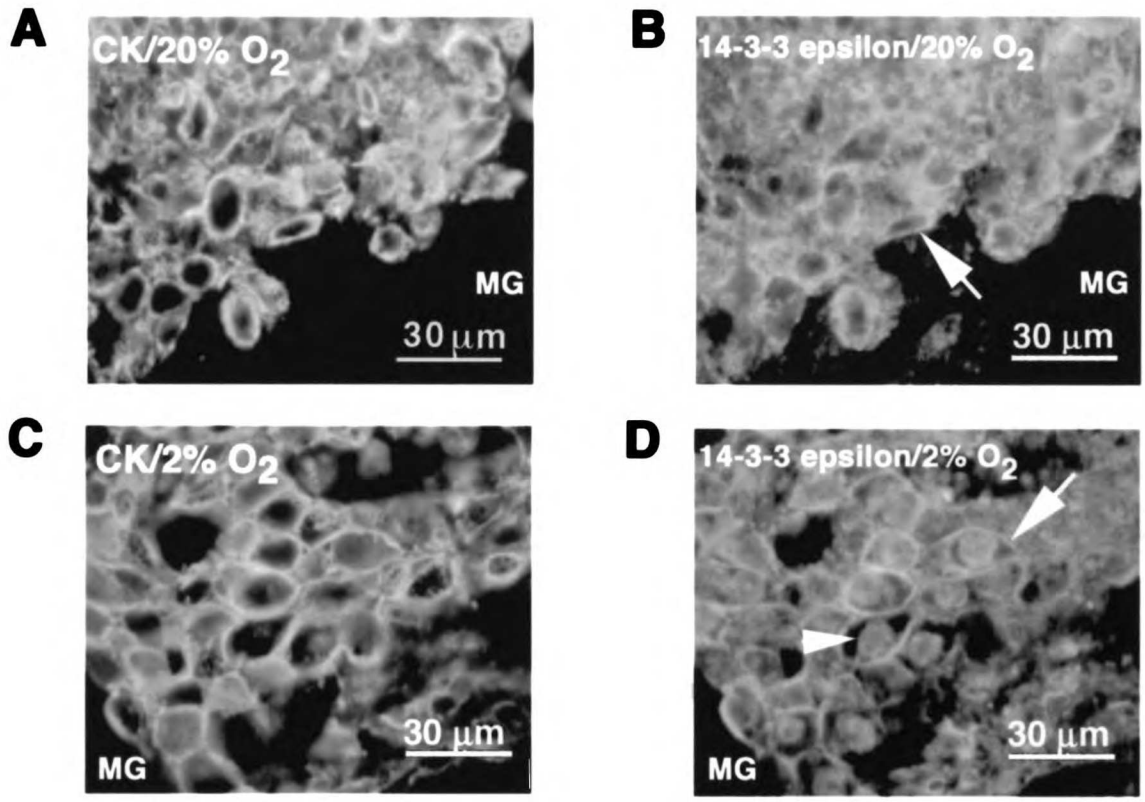


FIGURE 13: Immunolocalization showed that hypoxia induced translocation of 14-3-3 epsilon to the nucleus in cytotropoblasts from cultured villi. Sections of villi cultured on Matrigel (MG) that were maintained in either 20% O_2 (A and B) or 2% O_2 (C and D) were stained with antibodies that specifically reacted with cytokeratin (CK), a trophoblast marker (A and C), and 14-3-3 epsilon (B and D). In 20% O_2, 14-3-3 staining primarily localized to the plasma membrane region of the cell (arrow), although some antibody reactivity was also detected in the cytoplasm. In hypoxia, 14-3-3 epsilon expression was detected in association with both the plasma membrane (arrow) and the nucleus (arrow head).

were maintained in 2% or 20% O_2 (data not shown). To our knowledge this is the first evidence that 14-3-3 proteins play a role in cellular responses to hypoxia.

Discussion

We performed an initial characterization of the proteome of first trimester human placental cytotrophoblasts that differentiated along the invasive pathway *in vitro*. We were particularly interested in these cells because of their unusual tumor-like ability to invade the uterus and their molecular mimicry of endothelium; both properties are key determinants of pregnancy outcome (Damsky and Fisher, 1998). Although the cytotrophoblast proteome has not been studied by the methods we employed, 2-D PAGE maps of proteins isolated from other cellular compartments of the human term placenta have been published. These include the microvillus membranes of a different trophoblast population—multinucleate syncytiotrophoblasts that cover the chorionic villi (see Chapter 1, Figure 2A and 2B). Although the identity of most of the spots was not determined, the overall pattern was very different from that of our cytotrophoblast map (Webb et al., 1985). In contrast, an approach very similar to the one we applied, 2-D PAGE combined with protein spot identification by mass spectrometry, was used to map the proteome of mitochondria isolated from whole term placenta (Rabilloud et al., 1998). Since the cytotrophoblast stem cell population is largely depleted at the time of delivery, this preparation likely reflects the protein composition of mitochondria from other placental cell types, primarily fibroblasts and syncytiotrophoblasts (see Chapter 1, Figure 2A and 2B). Thus, it is not surprising that the cytotrophoblast proteome was also distinct from that of placental mitochondria. This result is in keeping with the 2-D PAGE database of radiolabeled mouse embryo proteins, including those of trophoblast cells (Latham et al., 1992). Although the identity of most of the gel spots is unknown, these maps demonstrate the specialized and changing nature of the trophoblast protein

repertoire during prenatal development. A similar picture has emerged from studies that used cDNA microarray technology to compare, at a global level, gene expression patterns in the mid-gestation mouse embryo and placenta (Tanaka et al., 2000).

The identification of protein gel spots in 2-D PAGE maps of cytotrophoblasts maintained under standard tissue culture conditions revealed high abundance proteins that are commonly found in all cells, as well as particular specializations. The former category included tropomyosin, cytokeratin and actin isoforms. Since term placenta is often used as a source of human proteins and RNA, the latter category included proteins that were already known to be expressed somewhere in this organ. However, only a subset has been localized to the specialized invasive cytotrophoblast population that was the subject of this study. For example, we were intrigued to find that abundant antioxidants included 1-Cys peroxiredoxin and AOE 37-2, in addition to Mn SOD, whose expression in human trophoblasts has been well studied (Church et al., 1992). Experiments to localize protein and mRNA have shown that 1-Cys peroxiredoxin is highly expressed in organs exposed to high oxygen levels, such as skin and lung (Kim et al., 1998; Novoselov et al., 1999). This finding correlates with the protein's function as an antioxidant. Interestingly, 1-Cys peroxiredoxin expression reduces phospholipid hydroperoxides and may thus play a vital role in defending the plasma membrane against the effects of oxidative stress (Fisher et al., 1999).

Similarly, we expanded the list of heat shock and chaperone proteins known to be expressed in cytotrophoblasts to include Grp-94, the endoplasmic reticulum homologue of HSP 90 that controls expression of the epidermal growth factor receptor (Supino-Rosin et al., 2000). Cyclophilin, which possesses enzymatic peptidyl-prolyl isomerase activity

that is essential for protein folding, was also abundant. Clearly, the annexins are also among the major cytotrophoblast proteins. Our work shows that annexin IV, which is expressed by epithelial cells (Dreier et al., 1998), is among other annexins whose expression in cytotrophoblasts has been previously described [e.g., annexin II; (Jensen and Matre, 1995)]. Finally, in keeping with the high glycogen content of these cells, enzymes that are involved in glycolysis are among the most abundant proteins in the cells.

We also examined the effects of hypoxia (2% O_2) on the first trimester human cytotrophoblast proteome. The reason for focusing on this variable was our past work showing that oxygen tension is an important regulator of cytotrophoblast differentiation/invasion. This relationship may reflect the fact that the placenta is the first organ to function during development. As a result, the initial stages of placental development occur before the conceptus accesses a supply of maternal blood [$\leq$ 10 wk of gestation; (Burton et al., 1999)]. In accord with this constraint, our previous work shows that cytotrophoblasts proliferate *in vitro* under hypoxic conditions that are comparable to those found during early pregnancy in the uterine lumen and the superficial decidua (2% O_2). As trophoblast invasion of the uterus and its resident blood vessels proceeds, the placental cells encounter increasingly higher oxygen levels (Pijnenborg et al., 1981), which trigger their exit from the cell cycle and subsequent differentiation (Genbacev et al., 1996; Genbacev et al., 1992). Very recently, we have explained the unusual response of cytotrophoblasts to hypoxia in terms of the known regulators of oxygen-dependent cellular responses. Specifically, hypoxia induces cytotrophoblast expression of the von Hippel-Lindau tumor suppressor protein which targets hypoxia-inducible factors for

ubiquitination and degradation (Genbacev et al., manuscript submitted). Thus, the placenta appears to have evolved highly specialized mechanisms for protecting itself against the usual spectrum of hypoxia-induced changes in cellular protein expression.

In accord with this observation, we found that hypoxia produced very few changes in the expression of cytotrophoblast proteins; some were anticipated and others could not have been predicted. As expected, we did see changes in the abundance of proteins that are involved in glycolysis and in mitigating the effects of oxidative stress. With regard to glycolysis, we observed an increase in the expression of triosephosphate isomerase and phosphoglycerate mutase. This result is consistent with hypoxia-induced increases in glycolysis that have been observed in other systems [including hepatoma (Li et al., 1996) and endothelial cells (Graven et al., 1994)], and with increased glucose consumption by human term cytotrophoblasts in response to hypoxia (Esterman et al., 1997). We also expected changes in the levels of antioxidants. In fact, the expression of two such proteins, Mn SOD and 1-Cys peroxiredoxin, decreased. The fall in SOD levels is consistent with our previous finding that the *in situ* expression of the Cu/Zn form of this enzyme is downregulated in invasive cytotrophoblasts in preeclampsia, a pregnancy complication that is thought to occur as a consequence of placental hypoxia (Many et al., 2000). Because of increasing evidence that reactive oxygen species (ROS) play a role in signaling pathways and gene transcription (Khan and Wilson, 1995; Palmer and Paulson, 1997; Servitja et al., 2000), identifying changes in the levels of the proteins which modulate levels of ROS may help to unravel pathways involved in the etiology of this disease.

Other changes in the observed cytotrophoblast response to reduced oxygen tension were unexpected. With regard to changes in protein abundance, we were surprised to see that the expression of both annexin II and tropomyosin were upregulated by hypoxia. Annexins are a family of calcium and phospholipid binding proteins with multiple functions (Raynal and Pollard, 1994). With regard to the consequences of increased cytotrophoblast expression of annexin II in hypoxia, several possibilities exist. In other cells, annexin II levels correlate with proliferative capacity (Menaa et al., 1999) (Chiang et al., 1999). Our results suggest that this relationship may also be true in cytotrophoblasts. Additionally, the ability of annexin II to control fibrinolysis in endothelial cells (Hajjar and Acharya, 2000) may suggest a parallel function in cytotrophoblasts which have many vascular-like properties. Clearly, limiting fibrin deposition at sites where cytotrophoblasts interface with maternal blood (see Figure 2A) is a critical determinant of the outcome of pregnancy. Likewise, an increase in tropomyosin expression in response to hypoxia has been observed in arterial endothelial cells, although the functional significance of this observation is not yet understood (Rao et al., 1994).

Finally, the relative abundance in cytotrophoblasts of certain members of the 14-3-3 protein family prompted us to use additional methods to search for hypoxia-induced changes in their expression. Immunoblotting analyses confirmed that both 14-3-3 epsilon and zeta migrated as single bands on 1-D gels and that their abundance was not influenced by oxygen tension. In contrast, immunolocalization showed that the epsilon, but not the zeta species, moved to the nucleus in hypoxia. Although the precise consequences of this translocation are not known, we assume that this observation is

indicative of oxygen-regulated interactions between 14-3-3 epsilon and a subset of proteins to which it binds. These include components of intracellular pathways that influence processes such as transcription (Brunet et al., 1999; Pan et al., 1999), passage through the cell cycle (Peng et al., 1997; Yang et al., 1999; Vincenz and Dixit, 1996; Waterman et al., 1998; Zha et al., 1996), signal transduction (Fu et al., 1994; Hausser et al., 1999; Muslin et al., 1996; Ogihara et al., 1997; Wakui et al., 1997), and cell adhesion (Garcia-Guzman et al., 1999; Gu et al., 1999).

In summary, this study focused on the proteome of first trimester human cytotrophoblasts. We obtained new data about their intracellular milieu by identifying relatively abundant proteins with potentially important functions, such as 1-Cys peroxiredoxin and AOE 37-2 that are involved in handling oxidative stress. These results, combined with information about the effects of hypoxia, give us new insights into how these unusual cells respond to the changes in oxygen tension that normally occur during the first trimester of pregnancy. In addition, a subset of these molecules could be used as markers to aid in unraveling the etiology of pregnancy complications that have been linked to placental hypoxia, studies now in progress.

Chapter 4: Salivary agglutinin is gp-340.

Summary

While the previous sections dealt with functional expression proteomics, that is identifying changes in protein expression while probing a system with different variables, the following two chapters pertain to the identification of proteins in biological complexes. In this chapter, we discuss our studies that identified the protein core of salivary agglutinin, a protein which binds to and aggregates various oral bacteria. Salivary agglutinin was identified to be gp-340.

Introduction

Human saliva has several critical functions, including lubrication (Aguirre et al., 1989; Gans et al., 1990; Hatton et al., 1985), digestion (Mandel, 1987), and formation of a bioactive semipermeable barrier (pellicle) that coats oral surfaces (Al-Hashimi and Levine, 1989; Bennick et al., 1983; Fisher et al., 1987). Saliva also acts to regulate the composition of the oral flora and is critical in maintaining oral health. Saliva fulfills this function by virtue of its antimicrobial activity (Levine et al., 1987; Obenauf et al., 1986). It also promotes selective microbial clearance or adherence (Gillece-Castro et al., 1991; Groenink et al., 1996; Murray et al., 1992; Scannapieco et al., 1995). The diverse functions attributed to saliva are allocated among its many components, which include amylases, cystatins, proline-rich proteins, proline-rich glycoproteins, carbonic anhydrases, peroxidases, statherins, histatins, lactoferrin, lysozyme, sIgA, mucins and salivary agglutinin. Protein sequences have now been deduced for all the major salivary

components except the agglutinin (Azen and Maeda, 1988; Bobek et al., 1993; Levine, 1993; vanderSpek et al., 1989).

Salivary agglutinin was identified as a protein fraction that mediates specific adhesion and aggregation of *Streptococcus mutans* (Carlén et al., 1996; Ericson and Rundegren, 1983; Kishimoto et al., 1989; Lamont et al., 1991). Monoclonal antibodies to agglutinin block adherence of *S. mutans* to experimental pellicles and aggregation of the bacterial cells by parotid saliva (Carlén et al., 1998; Carlén and Olsson, 1995). Several studies have related the levels of agglutinin in saliva to the numbers of *S. mutans* in dental plaque (Carlén et al., 1996; Emilson et al., 1989), the rate of plaque formation (Magnusson et al., 1976), and the susceptibility to dental caries (Rosan et al., 1982). Other studies have not found these associations (Lenander-Lumikari et al., 1992; Tenovuo et al., 1992).

In spite of a potentially important role of agglutinin in regulating the composition of the oral flora, very little is known about the chemical nature of the molecule. When isolated from parotid saliva, it behaves as a 5 x 10^6 Da oligomeric complex which contains a major 440 kDa glycoprotein (Ericson and Rundegren, 1983). Immunoblotting experiments show that this protein is recognized by the same mAb that blocks *S. mutans* adherence and its parotid saliva-induced aggregation *in vitro* (Carlén and Olsson, 1995; Ericson and Rundegren, 1983). SDS-PAGE shows that the complex contains other proteins, including secretory IgA, and minor, as yet unidentified, components (Ericson and Rundegren, 1983; Kishimoto et al., 1989; Oho et al., 1998).

Although the bacterial aggregation properties of agglutinin have been studied, nothing is known of its amino acid sequence. We isolated the glycoprotein and analyzed

its peptide portion by mass spectrometry. The results showed identity with gp-340 discovered in airway secretions by virtue of its affinity for surfactant protein D (Holmskov et al., 1997; Holmskov et al., 1999).

Materials and Methods

Materials. High purity trypsin was from Promega (Madison, WI). Fast-stain® (Coomassie blue) was from Zoion Biotech (Shrewsbury, MA). α-cyano-4-hydroxycinnamic acid was obtained from Hewlett-Packard (Böblingen, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Collection of Saliva and SDS-PAGE. Human parotid and submandibular/sublingual (SM/SL) salivas were collected as the ductal secretions as previously described (Bratt et al., 1999; Gillece-Castro et al., 1991). The samples were either used immediately or mixed with an equal volume of loading buffer and stored at -20°C before the experiment. Parotid and SM/SL saliva samples, purified salivary agglutinin preparations and purified lung gp-340 were electrophoretically separated on polyacrylamide gels (Laemmli, 1970). The gels consisted of a 3% stacking gel and 10% running gel. Protein bands were visualized by staining with Fast-stain®. Dr. Akraporn Prakabphol collected the saliva and did the SDS-PAGE.

Biochemical Purification of Salivary Agglutinin. Four hundred µl of fresh parotid saliva from one donor was concentrated to 25 µl by using a Centricon-Plus 20 filter (Millipore, Marlborough, MA), then mixed with an equal volume of loading buffer. The concentrated sample was electrophoretically separated; the stacking gel contained 3% acrylamide and the running gel contained 10% acrylamide. After electrophoresis, the protein bands were visualized with Fast-stain®. Under these conditions the salivary

agglutinin was well separated from other proteins. This allowed excision of the M_r ~350 kDa band. This was done by Akraporn Prakabphol in Susan Fisher's laboratory.

Mass Spectrometry of Peptides Isolated from the Biochemically Purified Agglutinin. The excised agglutinin band was macerated and washed with 25 mM ammonium bicarbonate/50% acetonitrile . After concentrating under reduced pressure in a SpeedVac Concentrator (Savant, Holbrook, NY), the samples were placed in a solution of high purity trypsin (0.05 μg/μl of 25 mM ammonium bicarbonate). The proteolytic digestion was allowed to continue at 37°C for 16 h. The resulting peptides were eluted from the gel with a solution of 50% acetonitrile and 5% TFA in distilled water and the volume was reduced in a SpeedVac Concentrator. First, the eluate was analyzed by MALDI-TOF MS. Portions of unseparated tryptic digests were cocrystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive Biosystems DE-STR MALDI-TOF mass spectrometer equipped with delayed extraction operated in the reflector mode.

Alternatively, peptide samples were subjected to HPLC separation prior to mass spectrometry. The apparatus was fitted with a Michrom Bioresources MagicMS C18 column (0.2 x 50 mm; 5 μm particle size; 200 Å pore size) which was equilibrated with 7% acetonitrile/0.1% TFA in H_2O. A flow rate of 1 μl/min was established by using an Eldex Micropro pump. Peptides were eluted isocratically for 10 min followed by a linear gradient (0.95%/min) to a final mobile phase composition of 63% acetonitrile/0.082% TFA in H_2O. One to 2 μl HPLC fractions were spotted directly onto a MALDI target with 1.5 μl of α-cyano-4-hydroxycinnamic acid. Peptide sequence information was obtained by PSD as previously described (Clauser et al., 1999).

The peptide mass and peptide fragment-ion data were used with the MS-Fit and MS-Tag programs, which are available on the World Wide Web (http://prospector.ucsf.edu), to search databases to determine peptide identity.

Results

Salivary agglutinin was isolated by electrophoretically separating whole parotid saliva on a SDS-PAGE gel (Figure 14). The band corresponding to the molecular weight of agglutinin was excised and subjected to an in-gel trypsin digest. After elution of peptides, an aliquot of the unseparated digest was processed for MALDI-TOF MS to give a peptide mass fingerprint (Figure 15). A total of 15 peptides and 14 unique sequences were obtained from the biochemically purified sample (Table 2). The sequence of 5 of these peptides was determined by MALDI-TOF/PSD (bold italic typeface in Table 2). A PSD spectrum and fragmentation of the peptide m/z 1459.8 is shown in Figure 16, together with the interpretation and deduced amino acid sequence. The peptide mass fingerprint and PSD fragment-ions (Table 2) were used for database searching. The results showed that the peptides were identical to regions found within both gp-340 and its splice variant DMBT1, both members of the scavenger receptor cysteine-rich protein family.

FIGURE 14. A sample of whole parotid saliva was separated on a 10% polyacrylamide gel. The band of 350 kDa, denoted by the arrow head, was excised and subjected to an "in-gel" trypsin digest, prior to analysis by mass spectrometry.

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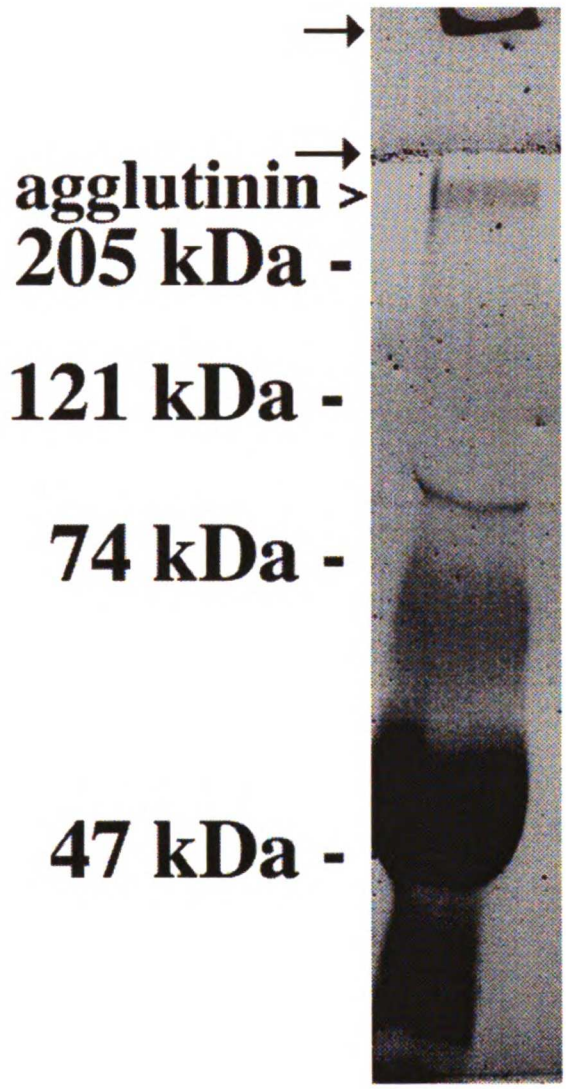

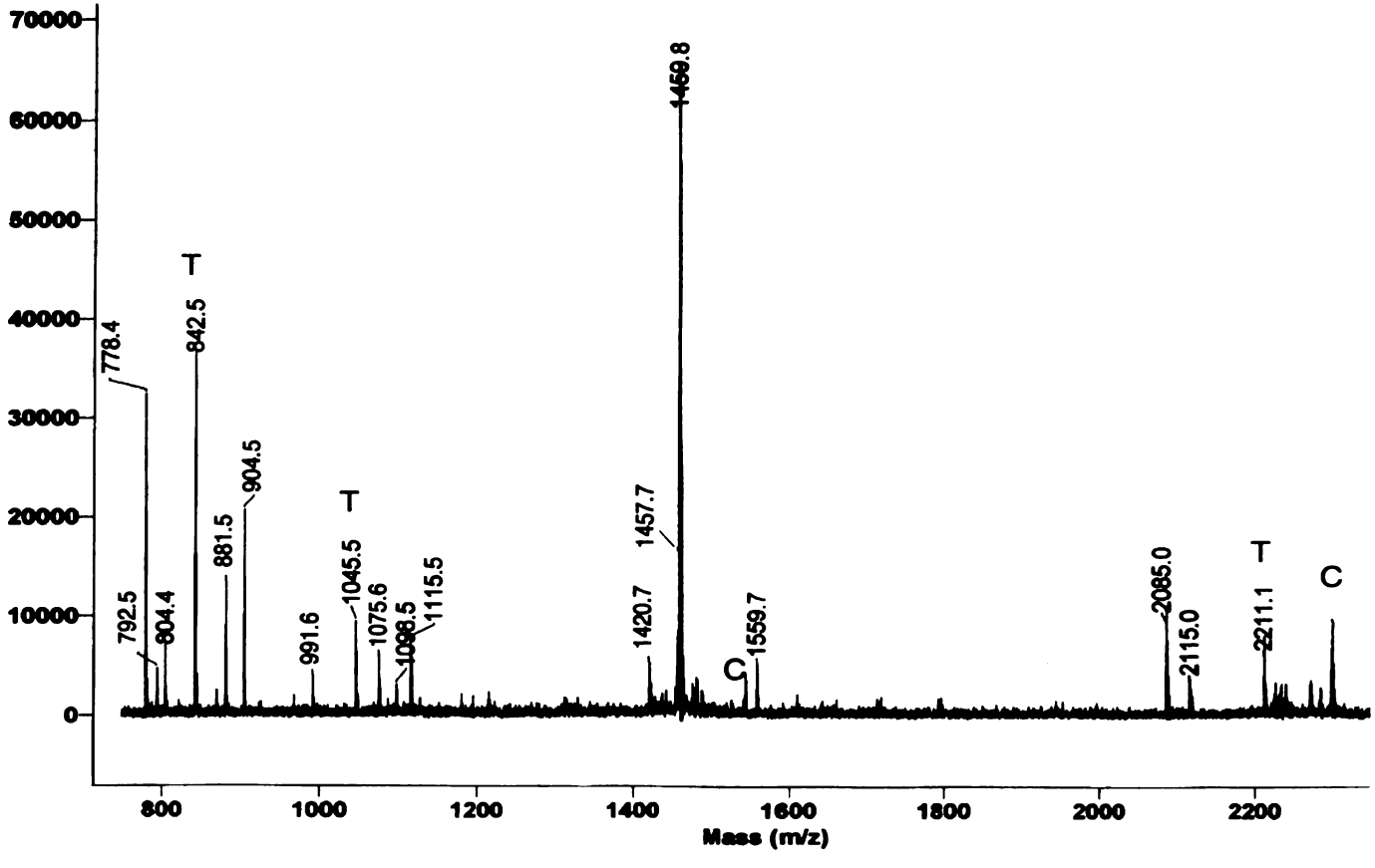

FIGURE 15. Peptide mass fingerprint of salivary agglutinin. A band corresponding to the estimated molecular mass of the salivary agglutinin was excised from an SDS-PAGE gel (see Figure 14) and digested with trypsin. MALDI-TOF on the unseparated digest resulted in the spectrum shown. C, peptides also present in a negative control, a portion of the polyacrylamide gel that did not stain for a protein band; T, trypsin autoproteolysis peptides used for internal mass calibration.

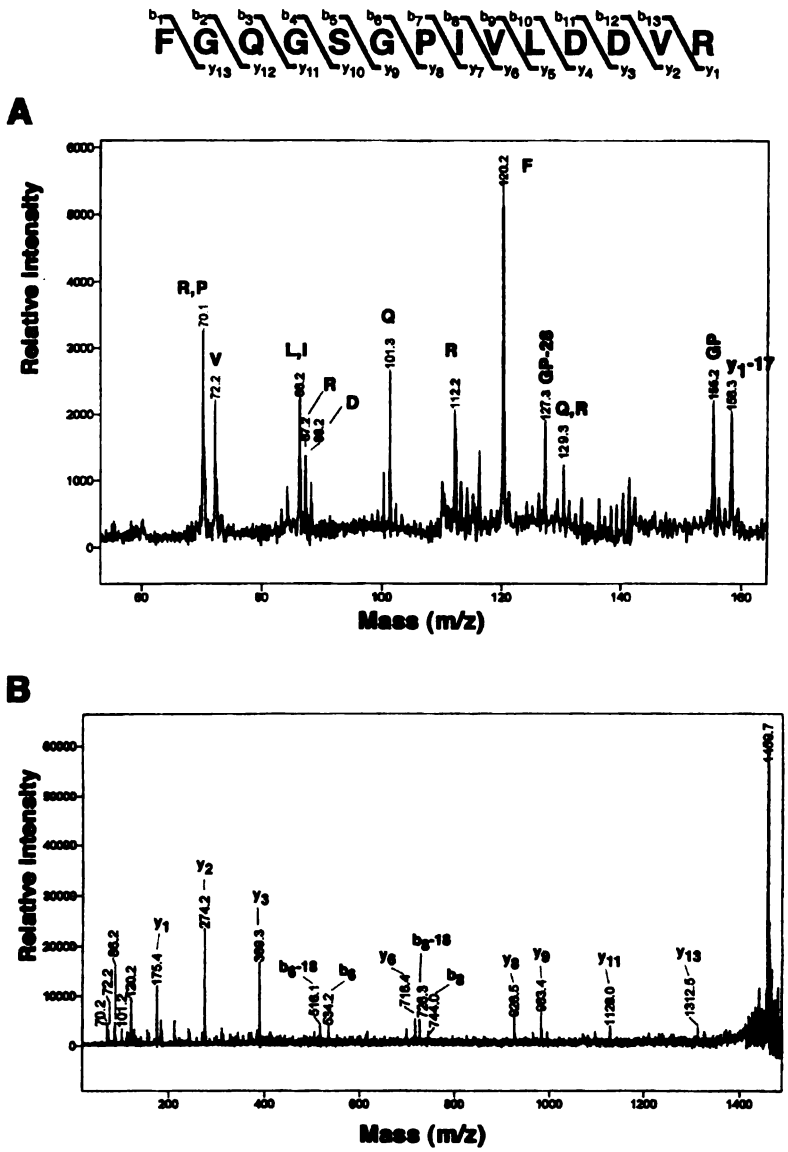

FIGURE 16. PSD spectrum of peptide m/z 1459. The lower mass region of the spectrum which includes immonium and related ions is shown in A. The remainder of the spectrum is shown in B. The fragment ions were used to search databases, which allowed assignment of the amino acid sequence shown above the spectrum. This sequence is identical to a region found within the scavenger receptor, cysteine-rich region of gp-340. The y- and b-ion series are labeled in the sequence.

TABLE 2. Summary of MS analyses of tryptic peptides from salivary agglutinin. The biochemically purified salivary agglutinin was subjected to in-gel trypsin digestion and an aliquot of the unseparated digest was analyzed by MALDI-TOF MS, which gave the mass of 15 peptides. The remainder of the digest was subjected to HPLC, and the separated peptides were analyzed by MALDI-TOF/PSD; the spectra showed the sequences of 5 peptides (bold italicized typeface).

Peptide sequence	Start-end[a]	MH^+ of tryptic peptides[b]	gp-340 domain
VEVLYR	247-252, 376-381, 507-512, 615-620, 746-751, 875-880, 1135-1140, 1264-1269, 1393-1398, 1653-1658	778.4	SRCR2, SRCR3, SDSR4, SRCR5, SRCR6, SDCR7, SRCR9, SRCR10, SRCR11, SRCR13
VEILYR	115-120	792.5	SRCR1
MTIHFR	1855-1860	804.4	CUB1
FPSVYLR	2353-2359	881.5	ZP
AFHFLNR	2346-2352	904.5	ZP
GRVEVLYR	245-252, 1651-1658	991.6	SRCR2, SRCR13
FISDHSITR	2099-2107	1075.6	CUB2
qIFTSSYNR[c]	1846-1854	1098.5	CUB1
QIFTSSYNR	1846-1854	1115.5	CUB1
GSFTSSSNFMSIR	2086-2098	1420.7	CUB2
DDTYGPYSSPSLR	2326-2338	1457.7	ZP
FGQGSGPIVLDDVR	418-431, 549-562, 657-670, 788-801, 917-930, 1048-1061, 1177-1190, 1306-1319, 1435-1448, 1695-1708	1459.8	SRCR3, SDSR4, SRCR5, SRCR6, SDCR7, SRCR8, SRCR9, SRCR10, SRCR11, SRCR13
QPGCGWAMSAPGNAR	1033-1047	1559.7	SRCR8
SAPGNAQFGQGSGPIVLDDVR	282-302, 1557-1577	2085.0	SRCR2, SRCR12
SAPGNAWFGQGSGPIALDDVR	150-170	2115.0	SRCR1

[a] Position of the amino acid residue in the deduced peptide sequence of gp-340.

[b] The mass accuracy of tryptic peptides was within ± 0.05 Da and masses listed are for the monoisotopic mass.

[c] q, pyro-glutamic acid.

Discussion

The primary structure of gp-340, recently established by molecular cloning, shows a polypeptide chain of 2413 amino acids (Holmskov et al., 1999). The *N*-terminus consists of a signal peptide and a sequence of 69 unique amino acids. Residues 95 to 1741 contain highly repetitive regions consisting of 13 scavenger receptor cysteine-rich (SRCR) domains. These domains are separated by SID (SRCR interspersed domain) sequences, of which there are 12. The interval between residues 1742 and 2134 contains a Ser-Thr-Pro-rich region and an additional SRCR domain flanked by two CUB (C1r/C1s Uegf Bmp1) domains, proteins modules initially found in complement subcomponents C1r/C1s, Uegf, and bone morphogenetic protein-1. The remaining residues contain a hydrophobic zona pellucida domain.

The 14 unique agglutinin peptides we detected by mass spectrometry were scattered throughout the entire protein and occurred within all the major domains (see Table 2). The identification of salivary agglutinin as the gp-340 protein was also suggested by the results of immunoblotting experiments with multiple antibodies specific for gp-340 (data not shown). The size of the salivary molecule was the same as that of gp-340 isolated from lung lavage, evidence that the agglutinin is the full-length molecule rather than the splice variant DMBT1, which lacks 628 amino acids consisting of 5 paired SRCR and SID domains (Holmskov et al., 1999; Mollenhauer et al., 1997). However, we have not ruled out the possibility that the salivary molecule is a differentially glycosylated form of DMBT1. Finally, the presence of this glycoprotein in saliva is consistent with reverse transcription-polymerase chain reaction analyses that show the

main sites of gp-340 expression are lung, trachea, salivary glands, small intestine and stomach (Holmskov et al., 1999).

Future studies will include determining the carbohydrate moieties of this glycoprotein. Knowing the structure of salivary agglutinin will greatly facilitate experiments to understand its various biological roles. One important general function likely arises from the glycoprotein's ability to interact with bacteria. The outcome of agglutinin binding may depend on the microbial ligand and its method of interaction, as well as whether the interaction occurs in solution, which probably favors clearance, or on oral surfaces where adherence becomes possible. In this regard, it is interesting to note that the agglutinin-binding adhesin AgI/II of *S. mutans* possess different domains that are involved in either agglutinin-mediated adhesion to hydroxyapatite surfaces or aggregation in solution (Brady et al., 1992). Finally, since gp-340 is an opsonin receptor for surfactant proteins A and D (Holmskov et al., 1999), future studies should investigate whether this interaction influences the bacteria-binding properties of the salivary molecule.

Dissecting the interactions between salivary agglutinin and various bacteria at a molecular level offers an interesting opportunity to restore, in the case of relevant disease states, the normal balance found in healthy individuals. Results from our lab show, an affinity of *H. pylori* for the salivary agglutinin. This supports the hypothesis of oral transmission which could include transient adherence in the mouth (Dunn et al., 1997). It is interesting that salivary mucins also interact with this bacterium (Prakobphol, Borén and Fisher; unpublished observations). The latter observation is in accord with two previous studies. First, it was shown that salivary mucins carried the fucosylated blood

group antigens (*i.e.* the ABO and Lewis type) (Prakobphol et al., 1993). Second, the Le^b and H-1 histo-blood group antigens mediate adherence of *H. pylori* to human gastric mucosa (Borén et al., 1993). Eradication of *H. pylori* infection with antibiotic treatment has proved to be difficult in patients who harbor *H. pylori* in the oral cavity/dental plaque (Miyabayashi et al., 2000). The results of this study suggest new, pharmacological strategies for inhibiting infections by this organism.

With regard to interactions with cells of the host immune system, work done in this lab has shown that the salivary agglutinin can carry the sLe^X epitope. This suggests that this molecule, like the low-molecular-weight salivary mucin (Prakobphol et al., 1999), could tether both bacteria and leukocytes, a potentially important consideration for immune interactions in the oral cavity. Also with regard to immune function, it is interesting to consider data that show gp-340 from lung stimulates random migration (chemokinesis) of alveolar macrophages (Tino and Wright, 1999). This activity could also enhance bacterial interactions with cellular components of the host immune system. Finally, studies by Nagashunmugam *et al.* suggest that salivary agglutinin has HIV-neutralizing properties. Thus, the agglutinin could be a component of the molecular system that prevents oral transmission of the virus. Now that we have determined the sequence of agglutinin, understanding the relationship between the structure of this protein and the many interesting functions it may carry out is the focus of future experiments.

Chapter 5. Calcineurin homologous protein (CHP) interacts with upstream binding factor (UBF), a nuclear transcription factor.

Summary

Mass spectrometry provides the sensitivity required to unravel complexes present in low abundance. In this project, immunoprecipitation was used to isolate proteins that interact with calcineurin homologous protein. After 1-D PAGE separation, the identity of an interacting protein was determined by mass spectrometry. MALDI-TOF/PSD analyses gave partial peptide sequences and a peptide mass fingerprint for upstream binding factor. This is the first example of the *in vivo* interaction of calcineurin homologous protein with a transcription factor.

Introduction

Calcineurin homologous protein (CHP) is a myristoylated Ca^{+2}-binding protein belonging to the EF-hand family of proteins. This 22 kDa protein is expressed in many tissues including eye, lung, liver, muscle, heart, kidney, thymus, and spleen (Lin and Barber, 1996). CHP is highly homologous to both calmodulin and the regulatory B subunit of calcineurin. This protein is implicated in many biological processes such as intracellular pH regulation (Lin and Barber, 1996), vesicular trafficking (Barroso et al., 1996), microtubule function (Timm et al., 1999), and T-cell activation (Lin et al., 1999). CHP's regulation of these multiple cellular pathways occurs through its interactions with other proteins. A few of CHP's protein partners have been identified. These include the

Na^+-H^+ exchanger (NHE1), a ubiquitously expressed plasma membrane transporter (Lin and Barber, 1996) and calcineurin (Lin et al., 1999), a widely expressed phosphatase.

The abundance of CHP is regulated by intracellular calcium levels; CHP levels decrease in Jurkat cells with increasing [Ca^{+2}] (Lin et al., 1999). Little, however, is known about the mechanisms or signaling pathways that regulate CHP. To better understand CHP's role in cell signaling and to study the mechanisms involved in the regulation of CHP expression, proteins that interacted *in vivo* with CHP were identified. By using a combination of immunoprecipitation and mass spectrometry, it was found that the transcription factor, upstream binding factor (UBF), interacts with CHP in a calcium sensitive manner.

Materials and Methods

Materials. High purity trypsin was from Promega (Madison, WI). Fast-stain® (Coomassie blue) was from Zoion Biotech (Shrewsbury, MA). α-cyano-4-hydroxycinnamic acid was obtained from Hewlett-Packard (Böblingen, Germany). ZipTips were acquired from Millipore (Marlborough, MA).

Immunoprecipitation and 1-D SDS-PAGE. Immunoprecipitations and 1-D SDS-PAGE were performed by Luanna Putney in Diane Barber's laboratory as previously described (Lin et al., 1999).

Mass Spectrometry. The bands of interest were excised, macerated and washed with 25 mM ammonium bicarbonate/50% acetonitrile. A blank gel piece that did not stain with Fast-stain® was also processed in parallel as a negative control. After drying under reduced pressure in a SpeedVac Concentrator (Savant, Holbrook, NY), the samples were placed in a solution of high purity trypsin (0.5 μg/μl of 25 mM ammonium bicarbonate). The proteolytic digestion was allowed to continue at 37°C for 16 h. The resulting peptides were eluted from the gel with a solution of 50% acetonitrile and 5% trifluoroacetic acid (TFA) in distilled water and the volume was reduced in a SpeedVac Concentrator. Samples were brought back to 15 μl with 1% TFA and desalted with ZipTips according to the manufacturer's instructions. Portions of unseparated tryptic digests were cocrystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive Biosystems DE-STR MALDI time-of-flight (TOF) mass spectrometer equipped with delayed extraction operated in the reflector mode.

Peptides were separated by high performance liquid chromatography (HPLC) prior to sequencing. The apparatus was fitted with a Michrom Bioresources MagicMS C18 column (0.2 x 50 mm; 5 μm particle size; 200 Å pore size) which was equilibrated with 7% acetonitrile/0.1% TFA in H_2O. A flow rate of 1 μl/min was established by using an Eldex Micropro pump. Peptides were eluted isocratically for 10 min followed by a linear gradient (0.95%/min) to a final mobile phase composition of 63% acetonitrile/0.082% TFA in H_2O. One to 2 μl HPLC fractions were spotted directly onto a MALDI target with 1.5 μl of α-cyano-4-hydroxycinnamic acid. Peptide sequence information was obtained by PSD as previously described (Clauser et al., 1999). MS and PSD spectra were interpreted to yield protein identities using the MS-Fit and MS-Tag programs (http://prospector.ucsf.edu) (Clauser et al., 1999).

Results

Hamster lung fibroblasts were cultured in the presence and absence of 2 mM $CaCl_2$. After lysing, polyclonal antibodies to CHP were used to affinity purify the protein and co-precipitating proteins were separated by SDS-PAGE. After staining with Fast-stain®, a doublet of proteins migrating at ~95 kDa co-precipitated with CHP in the absence, but not in the presence of calcium (Figure 17). This was done by Luanna Putney in the Barber Laboratory.

The two bands were excised and subjected to an overnight in-gel trypsin digest. Tryptic peptides were eluted from the gel, desalted with ZipTips, and analyzed by MALDI-TOF to obtain a peptide mass fingerprint. The two bands from the doublet gave the same fingerprint (Figure 18). A total of 6 peptides were detected by MALDI-TOF MS. Next, peptides were separated by HPLC and the sequence of peptide m/z 1504.7 was obtained by PSD. The spectrum and interpretation are shown in Figure 19. The peptide sequence and mass fingerprint of the two unknowns corresponded to UBF.

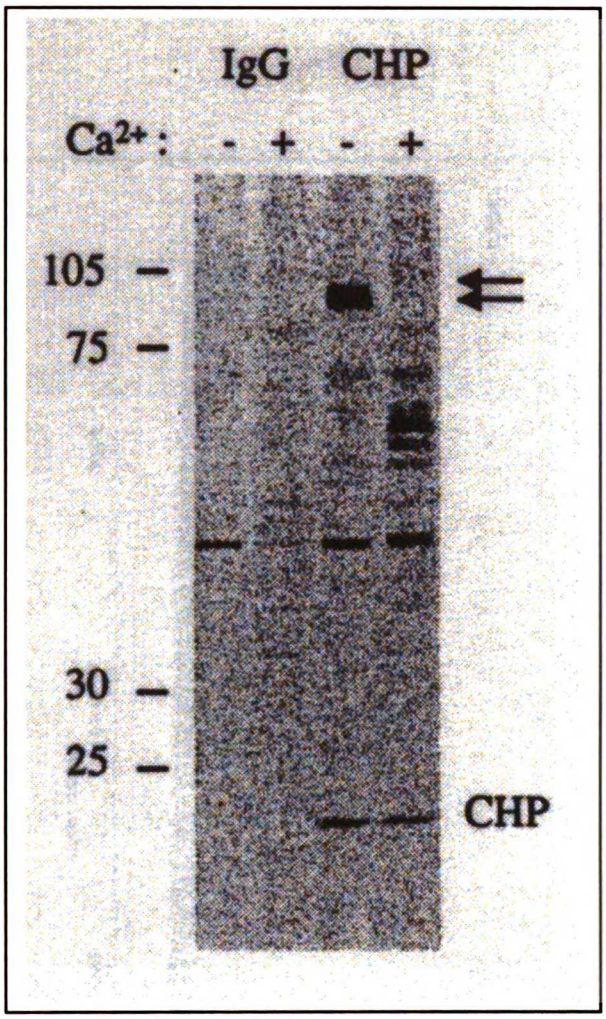

FIGURE 17. Calcineurin homologue protein (CHP) coimmunoprecipitates with a doublet (as shown by double arrows) in the absence of 2 mM $CaCl_2$. Control IgG does not coimmunoprecipitate with this protein.

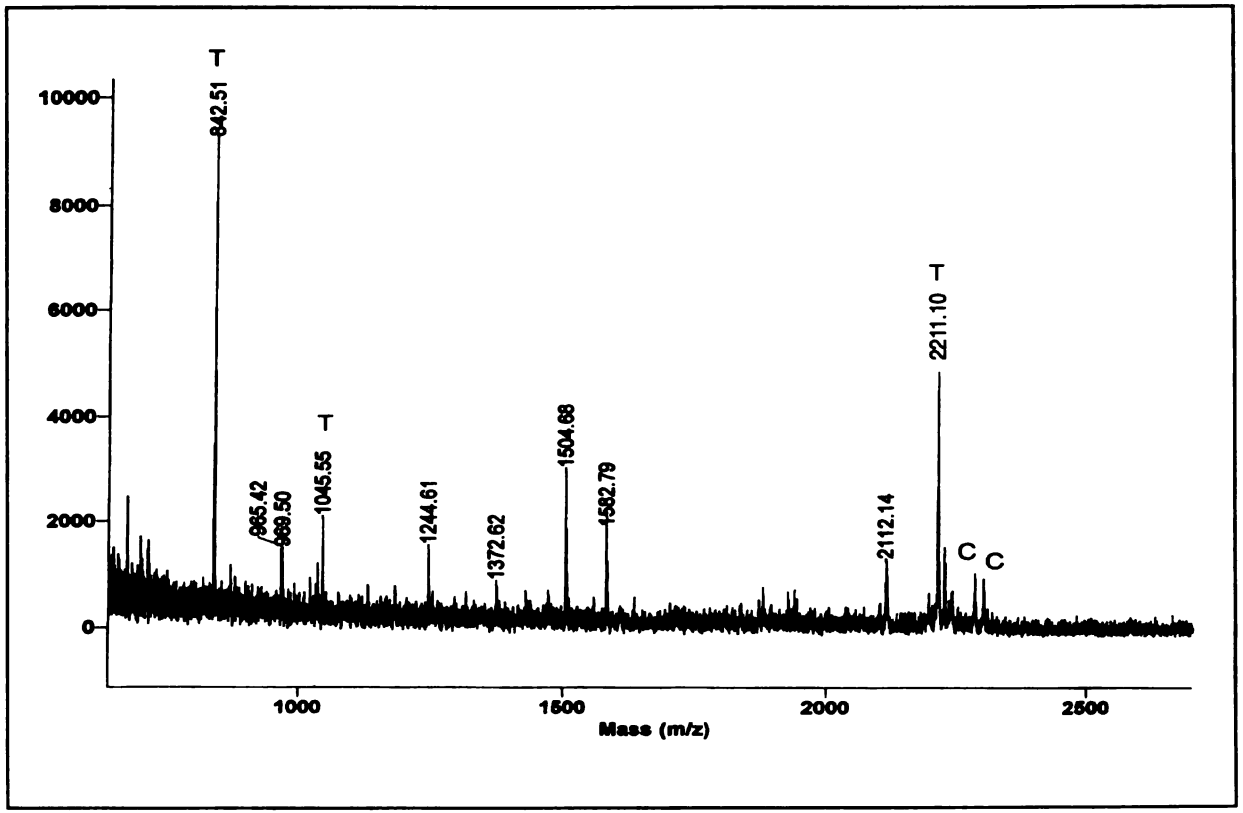

FIGURE 18. Peptide mass fingerprint of UBF. After an overnight in-gel trypsin digest of two unknown proteins that coprecipitate with CHP, MALDI-TOF MS on the unseparated digest was done. The resulting spectrum is shown. C, peptides also present in a negative control, a portion of the polyacrylamide gel that did not stain for a protein band; T, trypsin autoproteolysis peptides used for internal calibration of peptide masses.

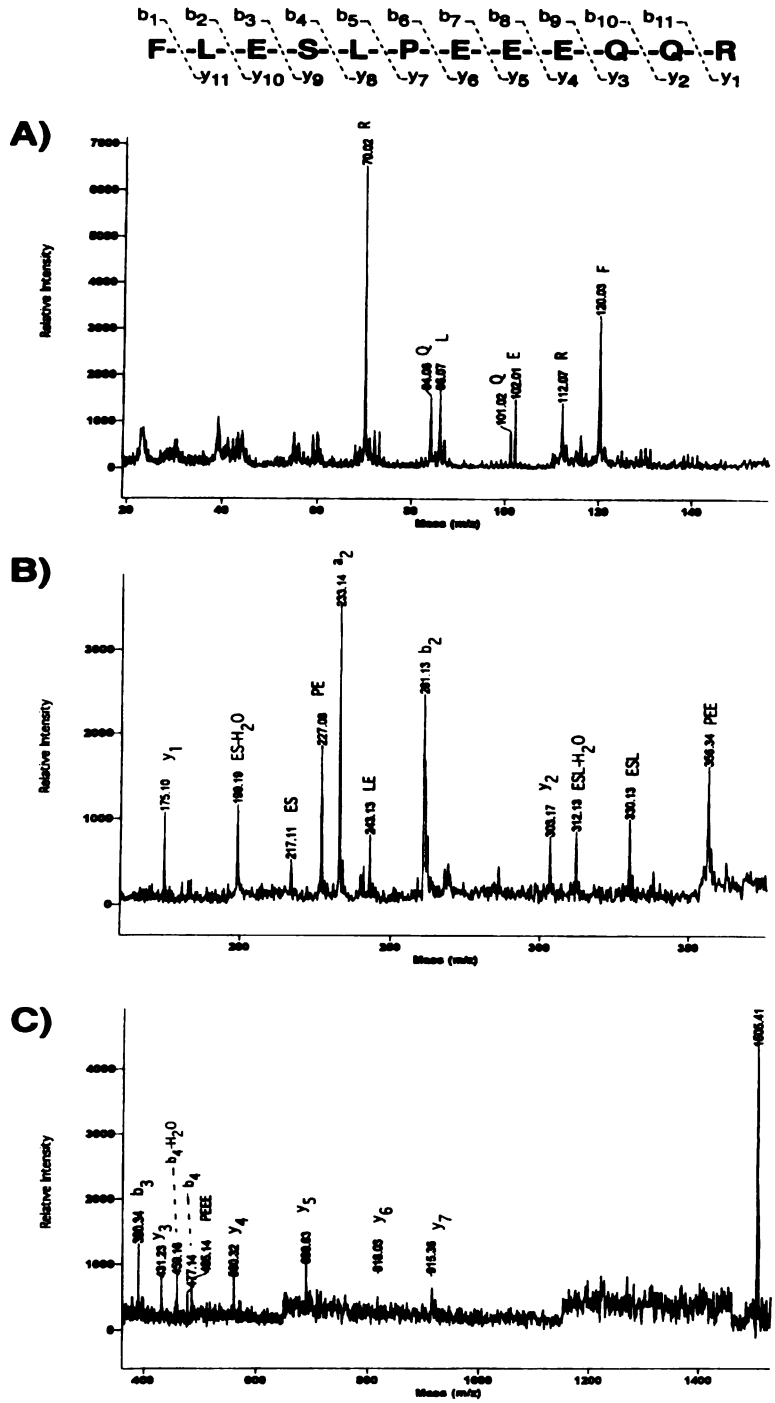

FIGURE 19. Postsource decay spectrum of peptide m/z 1504.7. (A) The low mass region of the PSD spectrum is expanded and shown. The amino acids present as indicated by the immonium and related ions are labeled. (B) and (C) show the remainder of the spectrum. Fragment ion types are indicated above peaks and the cleavage sites in the y- and b-ion series are labeled. The fragment ions were used to search databases, which allowed assignment of the amino acid sequence shown above the spectrum.

Discussion

UBF, a nuclear transcription factor required for basal activation of RNA polymerase I transcription of ribosomal RNA genes, plays a critical role in ribosome synthesis and cell proliferation (Klein and Grummt, 1999). This interplay between cell cycle progression and UBF could be mediated through UBF phosphorylation by a cyclin/cyclin dependent kinase (cdk) (Voit et al., 1999). Also, it has been shown that the protein encoded by the retinoblastoma tumor suppressor gene (Rb), which prevents progression through the cell cycle by its interaction with the E2F family of transcription factors (Harbour and Dean, 2000), can interact with UBF to repress the transcription of ribosomal RNA (Cavanaugh et al., 1995).

This is the first time that CHP has been shown to interact with a transcription factor and raises the possibility that CHP's function is mediated through transcription regulation. Interestingly, overexpression of CHP in fibroblasts inhibits cell proliferation and may be regulated by its interaction with UBF (L. Putney and D. Barber, unpublished observations). The connection between CHP, UBF, and cell proliferation is currently under investigation in the Barber laboratory.

Chapter Five. Conclusion

A valuable source for reproductive biologists has been developed with the determination of the protein expression profile of human cytotrophoblasts under standard culture conditions. These gels serve as a reference map of proteins from whole cell lysate to which future studies on human cytotrophoblasts can be compared. The development of organelle protein profiles of human cytotrophoblasts such as 2-D gel protein maps of nuclear proteins in the future would also be of benefit. These maps could possibly aide in protein translocation studies.

We have used these 2-D gels in the determination of the global effects of hypoxia on the suite of normal cytotrophoblast proteins. Hypoxia serves as an *in vitro* model for pregnancy complications such as preeclampsia which are believed to arise from reduced cytotrophoblast invasion of the uterus resulting in lowered oxygen tension at the maternal-fetal interface. Whether the same effects are seen with preeclampsia remains to be ascertained and will constitute the future directions taken in these studies.

Because the placenta is believed to contribute significantly to the development of preeclampsia, factors secreted by the placenta may be involved in promoting the maternal symptoms of preeclampsia (*e.g.* altered vascular function) (Ashworth et al., 1999; de Groot et al., 1998; McCarthy et al., 1993). Previous experiments suggest that conditioned media from cytotrophoblasts cultured under hypoxia can result in an altered response to vasoactive substances in isolated arteries similar to that seen in preeclampsia (Aalkjaer et al., 1985; Gratton et al., manuscript in preparation). This remains an open area for future proteomic investigations. What are the molecules secreted by the preeclamptic placenta which may result in these observed changes? Do cytotrophoblasts or placental villus

explants maintained under hypoxia also secrete the same suite of proteins? Similar approaches such as 2-D gels or isotope coded affinity tag-like chemistry and MS can be utilized in answering these questions.

As technologies and instrumentation improve, increased sensitivities will be gained in many aspects of proteomic studies. This should facilitate protein identification of less abundant proteins. For example, it will become increasingly easier to identify components of low-level complexes such as the CHP/UBF complex discussed previously. Finally, as the field of proteomics grows, protein identification will require less time and become readily accessible to more scientists.

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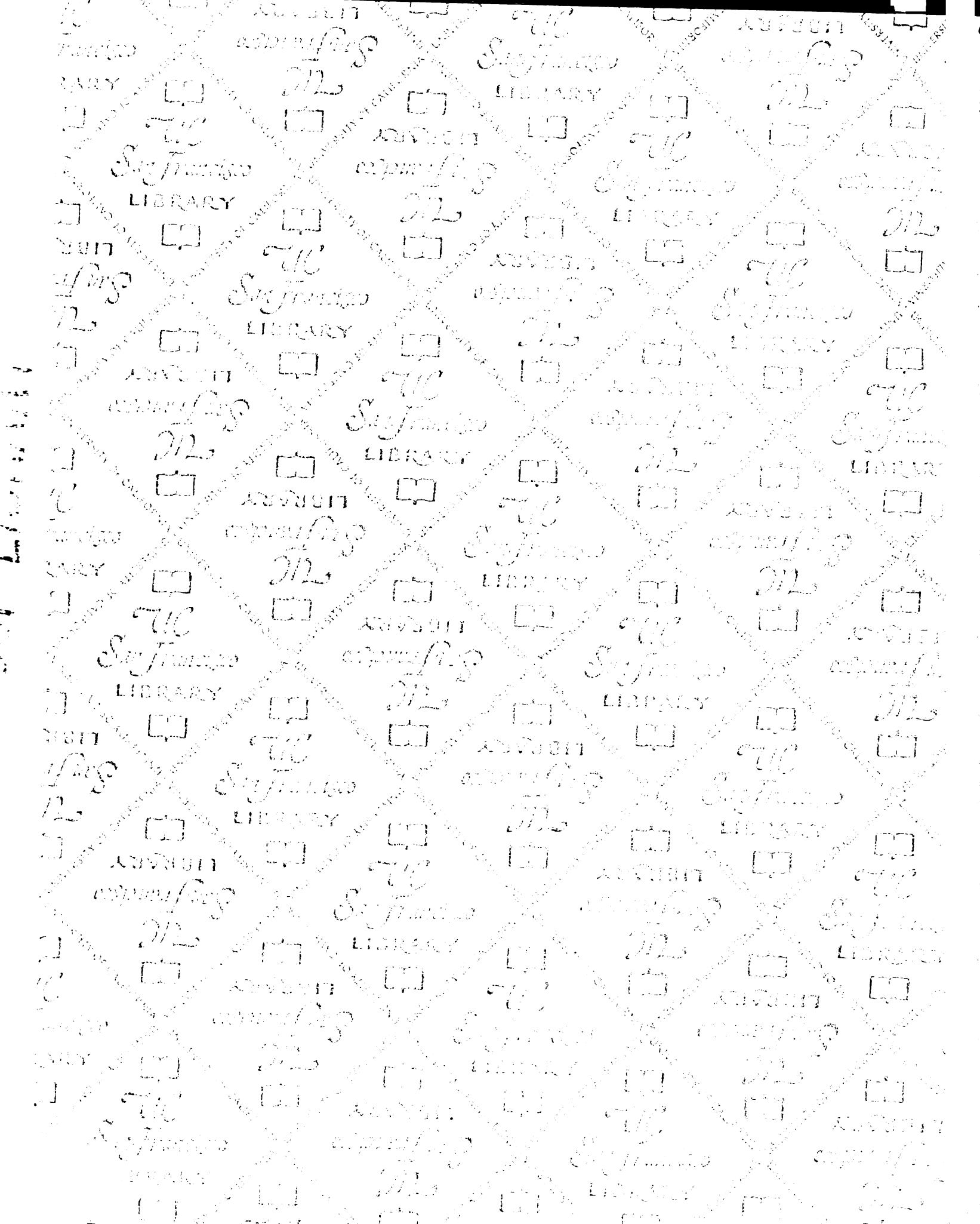
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